

**Bohn, Brent**

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**From:** Cowden, John  
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Hi Christy,

Happy Tuesday! I hope that things are going well for you today.

Here's a copy of the ADP. It's currently being revised, so take the text with a grain of salt! ☺

Let me know if you have any questions. Have a great afternoon!

John

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**Bohn, Brent**

1586

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I don't know if you have seen these; the Flora paper seems to be particularly useful.

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## Review Article

## Arsenic-induced oxidative stress and its reversibility

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## ABSTRACT

This review summarizes the literature describing the molecular mechanisms of arsenic-induced oxidative stress, its relevant biomarkers, and its relation to various diseases, including preventive and therapeutic strategies. Arsenic alters multiple cellular pathways including expression of growth factors, suppression of cell cycle checkpoint proteins, promotion of and resistance to apoptosis, inhibition of DNA repair, alterations in DNA methylation, decreased immunosurveillance, and increased oxidative stress, by disturbing the pro/antioxidant balance. These alterations play prominent roles in disease manifestation, such as carcinogenicity, genotoxicity, diabetes, cardiovascular and nervous systems disorders. The exact molecular and cellular mechanisms involved in arsenic toxicity are rather unrevealed. Arsenic alters cellular glutathione levels either by utilizing this electron donor for the conversion of pentavalent to trivalent arsenicals or directly binding with it or by oxidizing glutathione via arsenic-induced free radical generation. Arsenic forms oxygen-based radicals ( $\text{OH}^\cdot$ ,  $\text{O}_2^{\cdot-}$ ) under physiological conditions by directly binding with critical thiols. As a carcinogen, it acts through epigenetic mechanisms rather than as a classical mutagen. The carcinogenic potential of arsenic may be attributed to activation of redox-sensitive transcription factors and other signaling pathways involving nuclear factor  $\kappa\text{B}$ , activator protein-1, and p53. Modulation of cellular thiols for protection against reactive oxygen species has been used as a therapeutic strategy against arsenic. *N*-acetylcysteine,  $\alpha$ -lipoic acid, vitamin E, quercetin, and a few herbal extracts show prophylactic activity against the majority of arsenic-mediated injuries in both in vitro and in vivo models. This review also updates the reader on recent advances in chelation therapy and newer therapeutic strategies suggested to treat arsenic-induced oxidative damage.

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## Contents

Introduction	258
Arsenic-induced reactive oxygen species generation	258
Arsenic methylation	259
Alterations in signaling pathways	261
Alterations in transcription factor	262
Alterations in cell cycle phases	262
Arsenic-mediated ROS-induced apoptosis	262
Altered mitochondrial activity	263
Alterations in enzyme activity	263
Effects of arsenic on the cellular defense mechanism	264
Biomarkers of arsenic-induced oxidative stress	264
Reactive oxygen species	264
DNA oxidation products	265
Lipid peroxidation	265

**Abbreviations:** GSH, glutathione; GSSG, glutathione disulfide; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; RNS, reactive nitrogen species;  $\text{O}_2^{\cdot-}$ , superoxide anion;  $\text{OH}^\cdot$ , hydroxyl radical;  $\text{O}_2$ , singlet oxygen; MMA<sup>V</sup>, monomethyl arsonate; MMA<sup>III</sup>, monomethyl arsonous; ODD, oxidative DNA damage; PDH, pyruvate dehydrogenase; XO, xanthine oxidase; ESR, electron spin resonance; 8-OHdG, 8-hydroxy-2-deoxyguanosine; ALAD,  $\delta$ -aminolevulinic acid dehydratase; GST, glutathione *S*-transferase; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; DMSA, *meso*-2,3-dimercaptosuccinic acid; MIADMSA, monoisoamyl-DMSA; MMP, mitochondria membrane potential; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; AST, aspartate aminotransferase; CAT, catalase; CK-2, casein kinase 2; RTK, receptor tyrosine kinase; NTK, nonreceptor tyrosine kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; PKC, protein kinase C; CDK, cyclin-dependent kinase; AP-1, activator protein 1; NF- $\kappa\text{B}$ , nuclear factor  $\kappa\text{B}$ ; GADD45, growth arrest and DNA damage 45; LDL, low-density lipoprotein; Nrf2, transcription factor NF-E2-related factor 2.

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Protein oxidation . . . . .	265
Other biological indicators . . . . .	265
Antioxidant levels . . . . .	265
Pathophysiology of arsenic-induced ROS-mediated diseases . . . . .	265
Hepatic and renal disorders . . . . .	265
Cardiovascular disorders . . . . .	265
Type 2 diabetes . . . . .	266
Neurological defects . . . . .	267
Carcinogenesis . . . . .	267
Use of antioxidants (synthetic or herbal)/chelating agents in reducing arsenic-induced oxidative stress . . . . .	268
Antioxidant supplementation (natural and synthetic) . . . . .	269
N-acetylcysteine (Scheme 1) . . . . .	270
$\alpha$ -Lipoic acid (LA) (Scheme 2) . . . . .	270
Vitamin E ( $\alpha$ -tocopherol) and vitamin C . . . . .	270
Taurine (Scheme 5) . . . . .	270
Quercetin (Scheme 6) . . . . .	271
Essential metals . . . . .	271
Natural/herbal antioxidants . . . . .	272
Chelation therapy . . . . .	272
Conclusion and future strategies . . . . .	273
Acknowledgments . . . . .	273
References . . . . .	274

## Introduction

In today's world, environmental and occupational surroundings can generate a variety of modes for exposure to various forms of metals. Common sources of metal exposure include groundwater contamination, leather tanning, and mining [1]. Even though heavy metals such as iron and copper in trace amounts are vital for normal biological functioning of cells, extensive exposure to certain heavy metals could be linked to cellular damage, inflammation, and cancer [1,2]. Arsenic (As) is one of the most widely studied elements in the field of metal intoxication after lead (Pb). Arsenic is a metalloid found in water, soil, and air from natural and anthropogenic sources and exists in inorganic as well as organic forms [3]. The major inorganic forms of arsenic ( $As_i$ ) include trivalent meta-arsenite ( $As^{3+}$ ) and pentavalent arsenate ( $As^{5+}$ ). Whereas As in surface water mainly exists as  $As^{5+}$ ,  $As^{3+}$  is more prevalent in deep anoxic wells. Trivalent arsenic is known to be more toxic than the pentavalent form [4]. Humans can be exposed to arsenic via air and food; the major exposure route of  $As_i$  is through contaminated drinking water, especially in India, Bangladesh, China, and some Central and South American countries [5]. Arsenic concentrations in drinking water in Argentina (200 ppb) [6,7], Mexico (400 ppb) [8,9], Taiwan (50–1980 ppb) [10], and the Indo-Bangladesh region (800 ppb) have been reported to be well above the WHO guidelines' maximum permissible value (10 ppb) [11]. Chronic arsenicosis due to drinking arsenic-contaminated water is reported to affect more than 200 million people worldwide, with approximately 38 million residing in the Indo-Bangladesh region [5,12,13]. Various reported epidemiological studies have linked arsenic intoxication with internal cancers [14–18], blackfoot disease [19], vascular diseases [20,21], and diabetes [22–24].

The mode of action of arsenicals is quite complicated, and to understand it, multifactorial determinants need to be addressed. These determinants range from physicochemical properties, such as the valence state (trivalent/ pentavalent), degree of methylation, charge at physiological pH, and electrostatic attraction and repulsion to active sites on important macromolecules, to pharmacokinetic factors (absorption, distribution, metabolism, protein binding, and excretion). Oxidative stress is currently the most widely accepted and studied mechanism of arsenic toxicity [25].

Thus, one of the major areas of current research interest has been to understand the mechanism of arsenic-induced oxidative stress

with the aim of finding a suitable, safe, and specific treatment using chelation therapy, alone or in combination with an antioxidant. In this review article, I attempt to highlight the various pathways that mediate arsenic-induced oxidative stress and the potential prophylactic and therapeutic measures employing strategies such as using antioxidants, chelation, or their combination.

## Arsenic-induced reactive oxygen species generation

Arsenic may induce oxidative stress by cycling between oxidation states of metals such as As, Fe, etc., or by interacting with antioxidants and increasing inflammation, resulting in the accumulation of free radicals in cells [26]. Major arsenic-induced ROS include superoxide anion ( $O_2^{\cdot -}$ ), hydroxyl radical ( $\cdot OH$ ), hydrogen peroxide ( $H_2O_2$ ), singlet oxygen ( $^1O_2$ ), and peroxy radicals.

Oxygen-derived radicals form a most important class of radical species generated in living systems because molecular oxygen that has a unique electronic configuration forms  $O_2^{\cdot -}$  by addition of one electron [27]. Superoxide anions, arising through metabolic processes or after oxygen "activation" by physical irradiation, are considered "primary" ROS. They can further interact directly, through enzyme- or metal-catalyzed processes, with other molecules to generate "secondary" ROS [2]. For instance,  $\cdot OH$  generated through superoxide-mediated process involving hydrogen peroxide plays an important role in mediating the genotoxic effects of arsenic [28]. Yamanaka and colleagues were the first to demonstrate arsenic-induced free radical formation [29,30]. Molecular oxygen reacts with dimethylarsine (a trivalent arsenic form and a minor *in vivo* metabolite of dimethylarsinic acid) to form dimethylarsinic radical and superoxide anion. Further, the addition of another molecule of molecular oxygen results in a dimethylarsinic peroxy radical and these arsenic radicals are known to be detrimental to cells [30]. Liu et al. also demonstrated arsenic-induced free radical formation in mouse livers [28].

Experimental results have shown the generation of  $O_2^{\cdot -}$  and  $H_2O_2$  after arsenic exposure in some cell lines such as human vascular smooth muscle cells [31], human-hamster hybrid cells [28], and vascular endothelial cells [32], whereas other cell lines such as HEL30 [33], NB4 [34], and CHOK1 [35] have shown induction of  $H_2O_2$ . Furthermore, arsenic-induced  $\cdot OH$  generation too has been reported in the striatum of rats [36]. Apart from the direct evidence of arsenic-induced ROS, indirect evidence too has been reported. For instance,

changes in antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) have been shown to suppress arsenic-induced sister chromatid exchanges in human lymphocytes [37]. Similarly  $H_2O_2$ -resistant Chinese hamster ovary (CHO) cells were resistant to arsenite, whereas CAT-deficient CHO cells were hypersensitive to arsenite insult, demonstrating arsenic-mediated ROS [38]. It is thus clearly evident that arsenic exposure results in ROS generation in various cellular systems; however, the source or mechanism involved remains unclear.

Mitochondria are suggested to be one of the important sites of ROS production (Fig. 1). The mitochondrial respiratory chain produces  $O_2$  (as a by-product) from the reaction of molecular oxygen with semiubiquinone. In addition to mitochondria, three other sources of ROS have been proposed: (i) Generation of intermediary arsine species may produce significant amounts of free radicals [39–41]. (ii) Methylated arsenic species can release redox-active iron from ferritin. Free iron plays a central role in generating harmful oxygen species by promoting the conversion of  $O_2^-$  and  $H_2O_2$  into the highly reactive  $^{\bullet}OH$  radical through the Haber–Weiss reaction [42]. (iii) ROS can be generated during oxidation of arsenite to arsenate [43,44].

### Arsenic methylation

Inorganic arsenic is known to reduce from  $As^{5+}$  to  $As^{3+}$  as a prerequisite for methylation in mammals. Although arsenic biotransformation has been heavily investigated, this discussion is restricted to the oxidative stress induced by the process or its metabolites. Reduction and oxidation between  $As^{3+}$  and  $As^{5+}$  take place in the plasma, whereas methylation reactions occur primarily in the liver [44,45]. Arsenic metabolism may follow two possible pathways, classical reduction and oxidative methylation and the novel glutathione (GSH) conjugation.

The classical pathway involves conversion of arsenite to monomethyl arsonate ( $MMA^V$ ) followed by monomethyl arsonous ( $MMA^{III}$ ), which enters second methylation to dimethyl arsenic acid ( $DMA^V$ ) with the end product dimethyl arsinous acid ( $DMA^{III}$ ). This process is catalyzed by a single enzyme, arsenic (3+) methyltransferase ( $As3MT$ ) [46]. GSH conjugation with  $As^{III}$  through  $As3MT$  is a multistep methylation process involving formation of arsenite triglutathione followed by monomethylarsonic diglutathione [ $MMA(SC)_2$ ] and finally dimethylarsinic glutathione [ $DMA(SC)_2$ ]. Whereas  $MMA(SC)_2$  may undergo spontaneous degradation to form  $MMA^{III}$  followed by  $DMA^{III}$ ,  $DMA(SC)_2$  may undergo degradation to form  $DMA^{III}$  to  $DMA^V$ , a major metabolite found in urine. Thus reduction may occur enzymatically, with  $As3MT$  and/or glutathione *S*-transferase Omega (GST), and nonenzymatically, in the presence of endogenous reductants such as glutathione and thioredoxin under favorable physiological conditions [47]. Accumulation of  $As_i$  and its methylated metabolites can shift significantly among target organs depending upon duration of exposure and dose [47].

The role of membrane transporters such as AQP9, glucose transporter 2 (GLUT2), and multidrug-resistant proteins (MRPs) in mediated arsenic uptake and efflux in methylation kinetics and the toxicity of arsenic has often been proposed. For instance, potential arsenic transporters such as AQP9 may assist in  $As^{III}$  uptake from the blood into hepatocytes, where it is metabolized by either of the pathways discussed above. The metabolites thus formed, i.e.,  $MMA^{III}$ , may be effluxed back to the bloodstream via AQP9, whereas GLUT2 and MRP2 help efflux GSH conjugates to the bile, preventing accumulation of arsenic and its metabolites in hepatocytes. Thus arsenic efflux through MRPs requires the formation of GSH adducts because inhibiting  $\gamma$ -glutamylcysteine synthase ( $\gamma$ GCS), a rate-limiting enzyme for GSH synthesis, and GST increases  $As^{III}$  toxicity. Further, NF-E2-related factor 2 (Nrf2) controls  $\gamma$ GCS, GST, and MRPs to play a

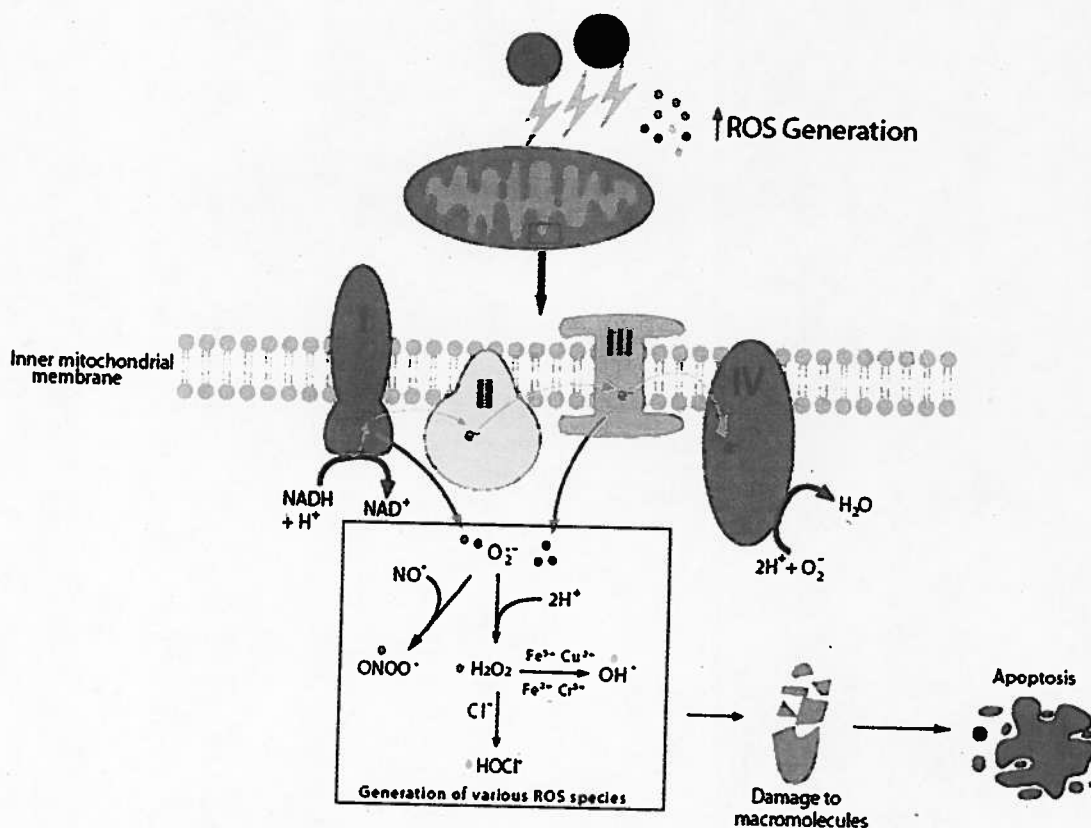
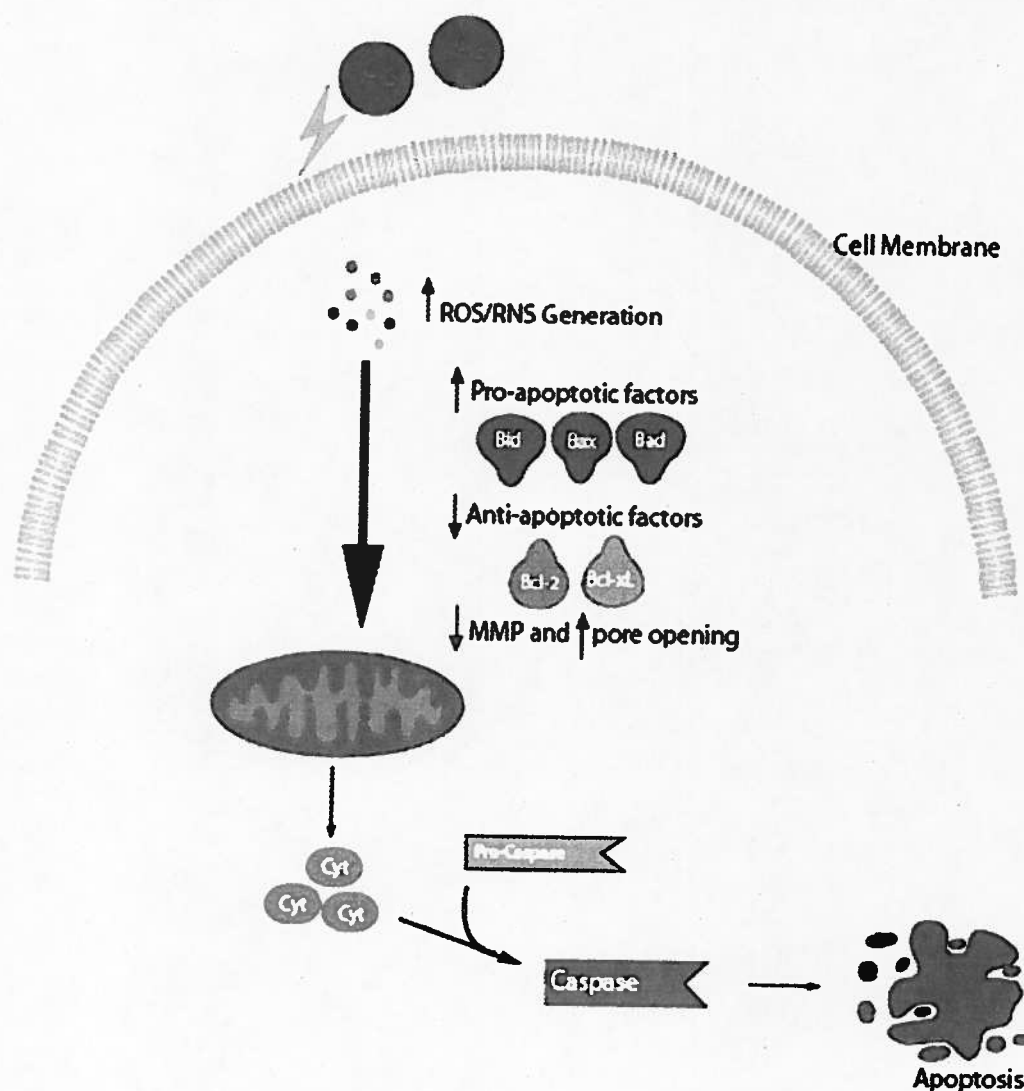


Fig. 1. Arsenic-induced mitochondrial generation of various reactive oxygen species and their effects. Arsenic induces significant ROS generation mainly through complex I and complex II of the electron transport chain (ETC). Superoxide radical generated through the ETC reacts with various other radicals present in the cell to form stable and long-lived reactive species, damaging macromolecules and inducing apoptosis via various pathways.

crucial role in arsenic efflux to the extracellular space, thus lowering cellular toxicity [48–52]. Arsenic methylation was previously believed to be a simple arsenic-detoxification reaction. However, increasing evidence suggests it to be a more complex metabolic process important in governing arsenic toxicity, because the toxicological profiles of arsenic metabolites vary *in vivo*. MMA<sup>III</sup> and DMA<sup>III</sup> have consistently been reported to be more toxic than any other As<sub>i</sub> metabolite, possibly because of their higher cellular uptake [49]. These are more potent than As<sup>III</sup> or As<sup>V</sup> in their ability to inhibit enzymes, inducing cell proliferation and causing DNA damage both *in vitro* and in intact cells; activate stress-mediated signal transduction in human cells; or inhibit insulin-stimulated signal transduction and insulin-dependent glucose uptake by adipocytes [48]. The majority of these toxic manifestations have been associated with ROS generation. Arsenic methylation is also involved in its genotoxic effects. Whereas arsenic-methylation-competent cells demonstrated significant oxidative DNA damage (ODD) due to arsenic exposure at 1  $\mu$ M for 5–18 weeks, methylation-deficient cells did not show similar effects even at 5  $\mu$ M exposure for 30 weeks. Furthermore, chemical inhibition of methylation by sodium selenite in methylation-competent cells abolished ODD and cells acquired a cancerous phenotype [53].

However, cells unable to methylate may still become cancerous, suggesting that there may be other mechanisms involved in arsenic-induced carcinogenicity. For instance, in humans, As3MT polymorphism is believed to mediate arsenic-induced carcinogenicity [54]. Production and excretion of DMA<sup>III</sup> have been linked to bladder carcinogenesis in rats exposed to DMA<sup>V</sup> [48]. In GSH-depleted cells, As<sup>5+</sup>, As<sup>3+</sup>, and MMA<sup>III</sup> showed increased toxicity; however, DMA<sup>V</sup> demonstrated reduced cytotoxicity, indicating that GSH is essential for DMA<sup>V</sup>-induced apoptosis [55]. It may be noted that GSH is an absolute requirement without any replaceable thiols for the human glutathione S-transferase Omega, the enzyme involved in a rate-limiting step for conversion of MMA<sup>V</sup> to MMA<sup>III</sup> (MMA<sup>V</sup> reductase) or DMA<sup>V</sup> to DMA<sup>III</sup> (DMA<sup>V</sup> reductase) [56,57]. Thus, GSH depletion due to utilization during arsenic metabolism may be crucial in arsenic-induced oxidative stress. Further, genotoxicity of MMA<sup>III</sup> has been attributed to its ROS-generating and direct DNA-damaging potential in human peripheral lymphocytes [58], PM2 cells, and HeLa cells. MMA<sup>III</sup>-induced malignant transformations have been correlated at biologically relevant concentrations. Chronic exposure to As<sub>i</sub> (1  $\mu$ M) and MMA<sup>III</sup> (0.05  $\mu$ M) caused malignant transformation of a human urothelial (UROtsa) cell line to anchorage-independent growth and



**Fig. 2.** Major events governing arsenic-induced mitochondrial-dependent apoptosis. Arsenic-induced ROS increase the expression of various proapoptotic molecules such as Bax and Bid and consequently decrease the expression of antiapoptotic molecules such as Bcl-2 and Bcl-XL. Simultaneously, ROS also cause calcium imbalance in the cells and decrease the activating caspase-dependent apoptosis.

tumorigenicity in SCID mice. These MMA<sup>III</sup> concentrations were within the range detected in human urine of chronically exposed subjects (0.06–0.09 and 0.04–0.4  $\mu$ M). Mechanisms suggested include the role of MMA<sup>III</sup> as a potent inducer of c-jun phosphorylation and activator protein 1 (AP-1) and its ability to bind with DNA, ERK activation in UROtsa cells, ROS-mediated damage, and alterations in signal transduction [59,60].

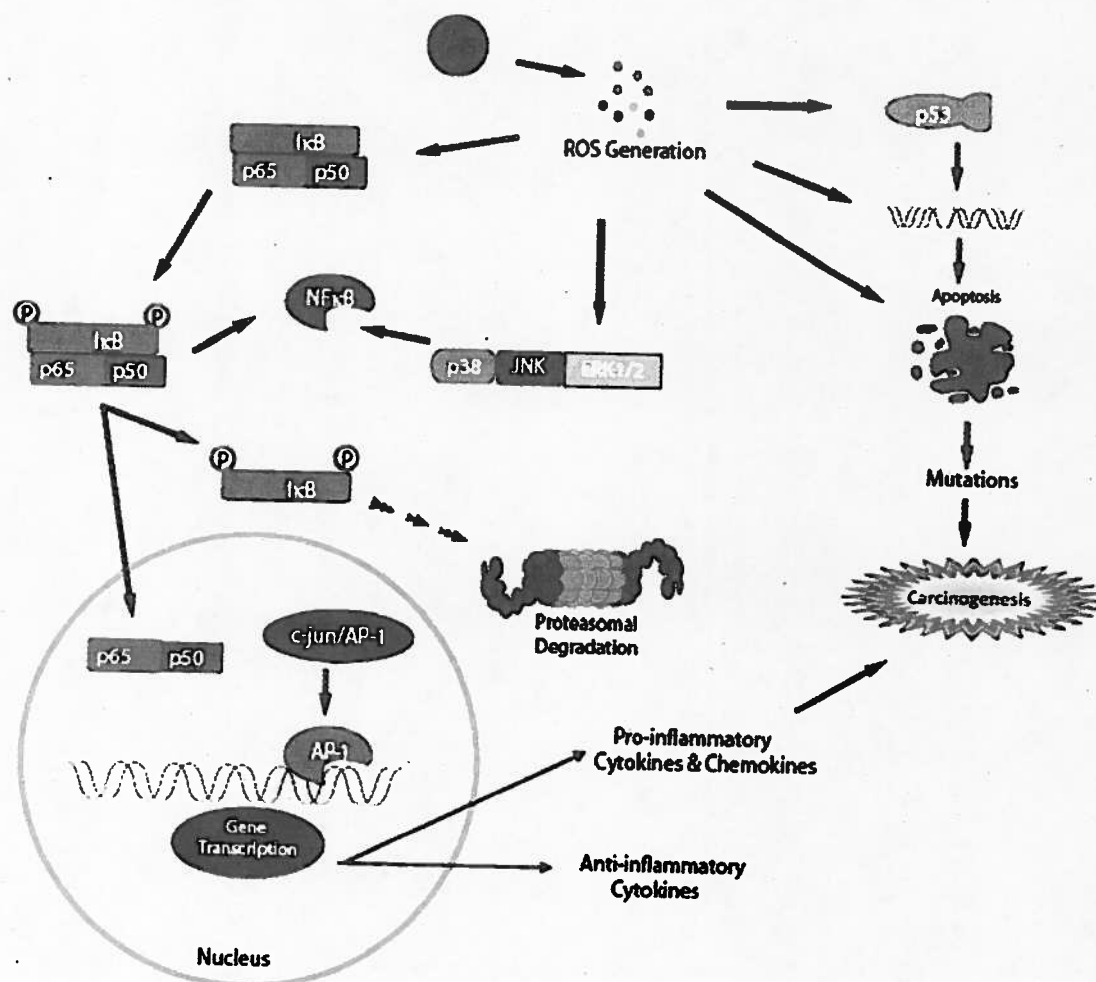
### Alterations in signaling pathways

In the recent past arsenic-induced interference and modulations in the signal transduction pathways have been revealed. However, arriving at a concrete inference from all the experimental information still remains the major challenge. Once arsenic gains entry inside the cell, through either phosphate transport proteins (arsenate) or aquaglyceroporin simple diffusion (arsenite), it may get metabolized in some cells, such as hepatocytes, but not in others (adipocytes, myocytes). Because arsenic toxicity is arsenic-species and cell-type specific, we may state that arsenic-induced ROS generation remains a common pivotal event. As ROS are critical in the signal transduction

pathways and transcription factor regulation, this review critically states in brief the oxidative stress-mediated pathway modulations. Major pathways affected include the tyrosine phosphorylation system, mitogen-activated protein kinases (MAPKs), and transcription factor families such as NF- $\kappa$ B and AP-1, described in a few recently published reviews [61,62] (Fig. 3).

The tyrosine phosphorylation system is mediated by a complicated interplay of two major types of protein tyrosine kinases: receptor tyrosine kinases (RTKs) and nonreceptor tyrosine kinases (NTKs). RTKs include growth factor receptors, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor, and vascular endothelial growth factor (VEGF) receptor. NTKs include members of the Src family [63]. Activation of EGFR causes the phosphorylation of the MAPK signal transduction pathway, a family of proteins significantly involved in cell apoptosis, differentiation, and transformation. MAPKs include three subgroups: extracellular signal-regulated kinase 1/2 (ERK1/2), stress-activated protein kinase/c-jun N-terminal protein kinase (SAPK/JNK), and p38 MAPK [61].

Arsenic elevates total cellular tyrosine phosphorylation in a dose-dependent manner with phosphorylation of EGFR as the prime target



**Fig. 3.** Arsenic-induced carcinogenesis and signaling pathways. ROS generated after arsenic exposure cause apoptosis directly or via the activation of the p53 pathway. Activation of the p53 pathway could lead to DNA damage resulting in apoptosis. Additionally, ROS activates I $\kappa$ B complex, which is phosphorylated by I $\kappa$ B kinase. This phosphorylation leads to the disassociation of I $\kappa$ B from p65 and p50. The former undergoes proteolytic degradation, and the latter migrate to the nucleus to activate various genes. ROS also activate various other molecules such as jun, p38, and ERK. All the above pathways directly or indirectly activate transcriptional factors such as NF- $\kappa$ B. These transcriptional factors along with others activate pro- or anti-inflammatory cytokines that could play important roles in carcinogenesis. The model presented here does not preclude the importance of other pathways involved in arsenic-triggered carcinogenesis.

[64–67]. The protein tyrosine phosphatases (PTPs; negative regulators of EGFR) contain a preserved thiol group that undergoes oxidation by ROS causing inactivation of PTPs. This inactivation leads to the transactivation of EGFRs without EGF binding [50]. Although there is no direct evidence suggesting the direct interaction of arsenic with EGFR, it is proposed that arsenic may interact with the –SH group of EGFR or indirectly via ROS to cause structural changes or dimerization [65,68]. Arsenic-induced activation of EGFR also recruits Shc and phosphorylates its tyrosine residue. This enhances interactions between Shc and growth factor receptor-bound 2 (Grb2), leading to the activation of downstream signaling molecules [66]. In vitro and in vivo studies have confirmed arsenic-induced activation of Shc along with EGFR and MAPK activation [69,70]. Similar to EGFR, arsenic-induced Shc activation is suggested via vicinal thiol groups or direct binding resulting in conformational changes or ROS generation [66]. Further, Shc may also serve as an important mediator in arsenic-induced ERK activation by serving as an adapter for the recruitment of Grb2 and Son of Sevenless. Thus, Shc in response to arsenic may activate downstream proteins such as MAPK; revealing parallel EGFR-dependent and -independent pathway [70].

Arsenic-mediated activation of MAPK signaling through the EGFR/MEK, EGFR/Ras/MEK, or Src/EGFR cascade has been identified in BEAS-2B cells, HaCaT cells, mouse epidermal JB6 cells, PC12 cells, Rat1 cells, and UROtsa cells [29–34,50]. ERK and p38 activation is suggested to be mediated by Ras/Raf/MEK pathways and JNK activation has been attributed to Rac, Rho, and MEKK3–4 [68]. However, EGFR-independent and MAPK-dependent arsenic-induced activation of transcription factors has also been reported in various cell types [38–40,50]. Alternatively, participation of protein kinase C (PKC) and not only ERK has been shown in the  $As_5$ -induced activation of AP-1 [41].

#### Alterations in transcription factor

As discussed in Section 4, arsenic interferes with signal transduction pathways that may be ROS mediated to induce transcription factors such as AP-1 and NF- $\kappa$ B. These stress-response transcription factors participate in early response and regulation of downstream target genes such as the proinflammatory genes involved in cellular defense. NF- $\kappa$ B plays a key role in cell proliferation and cell–cell interactions. It is inactive in the cytoplasm, being bound by its inhibitor I $\kappa$ B, which is commonly regulated by the IKK family, which itself is activated by protein kinases such as MEKK1, Akt, NIK, NAK, and PKC [61]. Stimulation induces the inhibitor to undergo degradation, rendering NF- $\kappa$ B able to translocate to the nucleus to activate transcription. Arsenic-induced ROS alter a large number of transcription factors such as AP-1, NF- $\kappa$ B, Nrf2, hypoxia-inducible factor 2, and others [71,72]. Effects of arsenic on NF- $\kappa$ B are dose/concentration, duration of exposure, and cell-type dependent. Whereas arsenic-induced ROS are considered the main cause of NF- $\kappa$ B activation at low doses (1–10  $\mu$ M), inhibition of NF- $\kappa$ B activity at high doses is mediated through IKK [73–76]. Up-regulation of NF- $\kappa$ B at low arsenic concentrations increased keratinocyte proliferation, whereas high doses enhanced caspase activity or AP-1 expression, inducing apoptosis via the Fas/Fas ligand (FasL) pathway [75–77]. Arsenic-induced NF- $\kappa$ B activation, although not fully revealed, has been suggested to be independent of I $\kappa$ B phosphorylation and subsequent degradation [78–80].

AP-1 is a complex protein composed of homodimers and heterodimers of oncogenic proteins of the jun and fos families [1]. Like NF- $\kappa$ B, the activity of AP-1 varies with arsenic dose and duration of exposure [81]. Whereas short-term exposure of arsenic enhances AP-1 binding to DNA via c-fos and c-jun, chronic exposure decreases AP-1's DNA binding ability, providing cells with some degree of tolerance to arsenic [82]. Interestingly, methylated forms of arsenic have greater effects on AP-1 than does  $As_5$  [81,83]. Recently, low doses of arsenic have been shown to alter cyclin D1 expression via c-jun/AP-1, causing cell transformation

[84]. Arsenic also activates p53 but this activation is oxidative state and chemical species specific and dose and cell-type dependent. For instance, arsenite ( $As^{3+}$ ) has the greatest ability to activate p53, whereas MMA has no effect [85]. Because activation of p53 initiates apoptosis through DNA damage, arsenic has also been used as a chemotherapeutic agent [86–88].

#### Alterations in cell cycle phases

Cell cycle is tightly regulated by a group of proteins called cyclins and cyclin-dependent kinases (CDKs). The G1, G2, and M phases have checkpoints that control the cell cycle and prevent late events from being initiated until the earlier events have been completed. Each checkpoint in turn is controlled via various signaling pathways. The G1 checkpoint is controlled through the p53-regulated signal pathways, involving p21 and cyclin E/CDK2, cyclin D/CDK4/6; the G2 through serine–threonine kinase ataxia telangiectasia-mutated protein (ATM)-regulated signal pathways, involving growth arrest and DNA damage 45 (GADD45), CDC25, and CDC2/cyclin B; and the M phase via microtubules [62].

Arsenic is known to induce cell cycle arrest, mostly at the G1 or G2–M phase [89]. Despite an incompletely known mechanism, in vitro studies in human cancer cell lines show arsenic-induced decrease in the levels of CDK/cyclin complexes [88,89]. GSH reduction in human pulmonary adenocarcinoma Calu-6 and lung cancer A549 cells by  $As_2O_3$ , causing growth inhibition by inducing G2/G2–M cell cycle arrest, has also been reported [90,91]. Although evidence suggests the potential role of p53 in arsenic-induced cell cycle arrest, results are rather variable [85,92–94]. A new GADD45 $\alpha$  isoform, GADD45 $\alpha$ 1, was reported recently in response to  $As_2O_3$  treatment. Unlike GADD45 $\alpha$ , GADD45 $\alpha$ 1 was unable to attenuate cell growth. Thus, overexpression of GADD45 $\alpha$  but not GADD45 $\alpha$ 1 sensitized cells to arsenic-induced prometaphase cell cycle arrest. Further, GADD45 $\alpha$ 1 may also be able to antagonize the function of GADD45 $\alpha$  in G2–M phase cell cycle arrest [84].

#### Arsenic-mediated ROS-induced apoptosis

Apoptosis is a critical cellular response to maintain normal cell development and proper function of multicellular organisms. There are two major signaling pathways of apoptosis, the death receptor pathway (extrinsic) involving tumor necrosis factor receptor, FasL, death receptors, and decoy receptors and the mitochondrial pathway (intrinsic) [95].

Gupta et al. investigated the apoptotic potential of arsenic in prostrate cancer cells as an investigational model [96]. Cheng et al. showed that arsenic-mediated cell death was not restricted to acute promyelocytic leukemia (APL) but holds to other malignant cells in vitro, including non-APL acute myeloid leukemia cells, myeloma cells, chronic myeloid leukemia cells, and solid-tumor cells of esophageal, prostate, ovarian, and neuroblastoma origin [97]. They also demonstrated that arsenic-induced Hep-2 cell apoptosis was modulated through GSH. Additionally, arsenic-induced apoptosis has been demonstrated in kidney macrophages, cervical cancer HeLa cells, pulmonary adenocarcinoma Calu-6 and A549 cells, calf pulmonary artery endothelial cells, human umbilical vein endothelial cells, and human pulmonary fibroblasts [98,99].

GSH plays an important role in apoptosis, which was found to have an inverse relationship [100]. Cells deficient in GSH biosynthesis when exposed to arsenite (0–2  $\mu$ M) show diminished Akt and c-fos levels and underwent apoptosis via ubiquitin–proteasome-mediated degradation. It is postulated that under GSH-deficient conditions, arsenic down-regulates heat shock protein (Hsp90) at both transcriptional and posttranscriptional levels through ATM kinase, checkpoint kinase 2, and p53-dependent activity leading to apoptosis [101]. Arsenic and MMA<sup>III</sup>-induced ROS-dependent degradation of

Hsp90 generates misfolded polypeptides that are functionally inactive, as well as accumulating as toxic species [102,103]. Woo et al. reported that sublethal doses of  $\text{As}_2\text{O}_3$  (0.05 M) sensitized CD95/Fas-induced apoptosis in human cervical cancer cells, which may be partly due to induction of ROS and subsequent up-regulation of CD95/Fas gene expression by NF- $\kappa$ B activation [104].

Arsenic-induced apoptosis via the mitochondrial-dependent pathway may initiate at low arsenic concentrations ( $\geq 10 \mu\text{M}$ ) in a human proximal tubule cell line (HK-2) [105]. The intrinsic apoptotic pathway is supported by arsenic-induced caspase protein and reciprocal regulation of Bcl-2/Bad with concomitant reduction of the mitochondrial membrane potential (MMP) and increased levels of cytosolic cytochrome c in *in vitro* and *in vivo* (10 mg/kg in rat) studies [102,106]. Our recent findings also revealed increased ROS causing  $[\text{Ca}^{2+}]_i$  imbalance, reduction of the MMP, and ultimately apoptosis with increased Bax expression in hepatic and neuronal cells *in vivo* after chronic arsenic exposure (25 ppm in drinking water for 4–6 months) [100,107] (Fig. 2). Further,  $\text{H}_2\text{O}_2$  plays the role of a mediator for arsenic-induced apoptosis via cytochrome c release, activation of CPP32 protease, and poly(ADP-ribose) polymerase (PARP) degradation [108,109]. 4-Hydroxynonenal (HNE), a major oxidative stress-induced membrane lipid peroxidation product, facilitates apoptosis through GSH depletion or enzyme malfunctioning via HNE-GSH/protein adduct formation. Thus, arsenic-induced anemia occurs through premature erythrocyte removal from circulation due to HNE-protein adduct formation that suppresses the antioxidant system and induces caspase-3 in erythrocytes [110]. Arsenic-induced ROS-mediated apoptosis is well documented in the literature, in which the mitochondria-driven apoptotic pathway is probably a more favorable mechanism for arsenic-induced cell death.

#### Altered mitochondrial activity

The mitochondrial electron transport chain is the main source of ATP generation in the mammalian cell. During energy transduction, a small number of electrons “leak” to oxygen prematurely, forming superoxide, which has been implicated in the pathophysiology of oxidative stress [111,112]. Complexes I and III, the main sources of superoxide generation (Fig. 1), release superoxide into the matrix and the extramitochondrial space respectively [113].

Mitochondria are the prime targets for arsenic intoxication, either indirectly via ROS accumulation or directly through condensing mitochondrial matrix and opening of permeability transition pores by virtue of its thiol-oxidizing property. In either case, arsenic-induced mitochondrial insult initiates apoptosis [114]. Because decreased MMP further generates ROS, it may be difficult to conclude whether arsenic-induced ROS are the cause of mitochondrial damage or a consequence of it. We reported that arsenic-induced mitochondrial damage in guinea pig liver leads to leakage of superoxide ions into the cytosol [100]. Although the source of superoxide production from either complex I or III could not be confirmed, Corsini et al. demonstrated that HEL30 cells incubated with complex I inhibitor completely abrogated the increased ROS induced by arsenite [33]. Disruption of the MMP brings morphological changes and causes loss of mitochondrial organization [115–117], which triggers a cascade of events such as release of cytochrome c, activation of apoptotic proteins (Bax, Bid), and down-regulation of Bcl-2, leading to apoptosis [100]. In chronically arsenic-exposed human subjects ( $204 \pm 102 \mu\text{g As/L}$  in drinking water and  $535 \pm 346 \mu\text{g As/L}$  in urine), mitochondria-dependent apoptosis was severalfold higher in peripheral blood mononuclear cells compared to those of normal individuals ( $6 \pm 1$  and  $27 \pm 11 \mu\text{g As/L}$ , respectively) [117]. Similar results were also observed in embryonic maxillary mesenchymal cells exposed to arsenic [118].

Arsenic-induced mitochondrial dysfunction has been attributed to multiple radical species, of which RNS, including peroxynitrites, play a

crucial role [119–121]. Peroxynitrite, formed by coupling of superoxide anion with NO, is a strong reactive isomer of nitrate anion ( $\text{NO}_3^-$ ) with longer half-life than  $\text{OH}^-$  [122]. Superoxide generated as a result of arsenic-induced mitochondrial damage may react with NO to generate peroxynitrite. In the absence of superoxide, peroxynitrite generation may occur with  $\text{NO}^-$  (reaction of NO with ferrocyclochrome c) and oxygen [123]. This peroxynitrite generation may be further enhanced by either  $\text{NO}$  or  $\text{O}_2^-$ , or decreased SOD activity, a common scenario post-arsenic exposure.

Mitochondria may also mediate the mutagenic effects of arsenic in mammalian cells. Liu et al. demonstrated that mitochondria are the direct targets of arsenic (1–2  $\mu\text{g/ml}$ )-induced genotoxicity in human-hamster hybrid cells [121]. It was proposed that the damage may be mediated through peroxynitrite, because mitochondrial-DNA-depleted cells and NO-synthase inhibitor did not respond to arsenic. Further, arsenic-generated peroxynitrite significantly up-regulated COX-2 and prostaglandin E2, the known mediators of inflammation in diseases such as atherosclerosis and coronary heart diseases [122].

#### Alterations in enzyme activity

Arsenic intensively affects the ROS-metabolizing enzymes called antioxidant enzymes, such as SOD, CAT, glutathione peroxidase (GPx), GST, and glutathione reductase (GR). Generally short-term exposure to low arsenic concentrations results in an increase in the activity of these enzymes, whereas chronic exposure usually results in their reduction. Arsenic is also known to regulate the activity of thioredoxin reductase, heme oxygenase reductase, and NADPH oxidase *in vitro* [108,124]. Arsenic is also known to alter activities of cytochrome P450s. There is emerging evidence that heavy metals could regulate CYP1A1 activity by enhancing aryl hydrocarbon receptors in a metal- and species-dependent manner [125]. Medina-Diaz and co-workers reported that arsenic and its metabolites increase CYP3A4 activity in a dose-dependent manner [126]. Arsenic is also shown to inhibit pyruvate dehydrogenase (PDH) activity either via oxidative damage or through binding to vicinal dithiols in both pure enzyme and tissue extracts. However, the arsenic concentration required to deactivate the enzyme is much lower than what is required for direct binding to thiol groups, suggesting an alternative mechanism [127].

Mice subchronically exposed to arsenic (4 ppm through drinking water for 60 days) had decreased gene expression of succinate dehydrogenase subunit A, ubiquinol-cytochrome c oxidoreductase, cytochrome oxidase (Cox6a2, Cox17), and ATP synthase (Atp5a1, Atp5g1, Atpif1) in brain cells [128], suggesting arsenic may also interact with these mitochondrial enzymes. In addition to mitochondria, arsenic binds to the molybdenum center of xanthine oxidase (XO), perturbing both oxidation–reduction potentials and the electron paramagnetic resonance signal. However, it does not disturb the oxidation–reduction potentials of iron–sulfur centers of the enzyme, but decreases the midpoint potential of the flavin adenine dinucleotide site. X-ray crystallography studies have shown the  $\text{AsO}_3$  moiety bound to the molybdenum atom of the active site through one of the oxygen atoms of arsenite-inhibited aldehyde dehydrogenase, a member of the XO family of mononuclear molybdenum enzymes [129].

NADPH oxidase is another important target for arsenic toxicity. Arsenic has been shown to induce up-regulation, phosphorylation, and membrane translocation of key subunits of NADPH oxidase [130–132]. Whereas up-regulation of NADPH oxidase via  $\text{p22}^{\text{phox}}$  has been linked to DNA damage in vascular smooth muscles [31] and actin filament reorganization, resulting in lamellipodia and filopodia [132],  $\text{p67}^{\text{phox}}$  and Rac1 subunits have been shown to play a prominent role in endothelial cells [133]. Further mutations in these subunits may result in reduced immunity and lead to immunocompromised congenital diseases such as chronic granulomatous disease [134,135]. Further, activation of NAD(P)H oxidase leads to generation

of superoxide anion as part of the oxidative burst of phagocytosis. The regulation and character of NAD(P)H oxidase are well reviewed by Griendling et al. [136].

The role of arsenic–protein binding has also been associated with carcinogenesis. Preferential sulfhydryl binding of arsenic and ability to competitively replace zinc allow it to inhibit zinc-finger-dependent enzymes. Zinc-finger (ZF) motifs are protein domains that maintain their three-dimensional structure by coordination of Zn(II), which binds at the cysteine binding site. Despite limited available literature, arsenic–ZF interaction is evident as a mechanistic basis for several effects, especially carcinogenesis. Arsenic may bind to tubulin; PARP-1; thioredoxin reductase; estrogen receptor- $\alpha$ ; arsenic (+3) methyltransferase; Keap1; GSH reductase; estrogen receptor- $\alpha$ ; glucocorticoid receptor; zinc-finger proteins of C2, C3, and C4 structure; transcription factors; metallothioneins; etc., *in vivo*. However, arsenic-induced ZF inhibition may also be mediated through oxidative stress. Moreover, because GSH might participate in zinc homeostasis, i.e., transfer of Zn(II) ion to targets such as ZF and proteins, arsenic-induced GSH depletion would have an indirect effect [137–139].

### Effects of arsenic on the cellular defense mechanism

Antioxidant response is the major cellular defense mechanism that a cell has against arsenic insult. Arsenic-induced antioxidant imbalance has been reported in numerous studies [2,140–143]. These studies have demonstrated that various enzymatic and nonenzymatic factors help in protecting cells by scavenging and clearing ROS [144]. The ability of glutathione, the most abundant nonprotein thiol in cells, to react with electrophiles directly or as a cofactor (for enzymes GPx and GST) may play an important role in arsenic detoxification and against arsenic-induced oxidative stress [49,145,146]. Chronic arsenic exposure (4–6 months; 25 ppm in drinking water), or very high dose, acute exposure (15.86 mg/kg, ip) causes GSH depletion in mice and rats, respectively [100,147,148]. This could be due to the utilization of GSH as electron donor for arsenic metabolism or direct binding due to thiol preference. For example, mice exposed to a lower arsenic dose of 3.2 ppm in drinking water for 2 months showed increased hepatic GSH level, whereas long-term exposure (4 months) decreased GSH in the same study [149]. Increase in GSH level thus indicates an adaptive mechanism of the cell to counter arsenic attack. Arsenic may induce GSH increase by facilitating GSH biosynthesis via up-regulated gene expression of glutamate–cysteine ligase subunits, as evident by an *in vitro* study in a murine hepatocyte cell line. This effect was inhibited by *N*-acetylcysteine (NAC), indicating a role for ROS [146].

GSH also modulates the redox status of specific thiol residues of arsenic-targeted proteins such as transcriptional factors, caspases, and stress kinases [145–150]. Arsenic-induced depletion of other antioxidant defense enzymes, such as SOD, CAT, and GPx, also demonstrates dose-, time-, and organ-dependent variability [100,151,152]. Again, where chronic exposure invariably results in decreased enzyme activity, such as that of hepatic CAT, GST, and GR after 9, 12, and 15 months of arsenic exposure, respectively, low concentrations (0.1–10  $\mu$ M) and acute exposure are generally reported to enhance enzyme activity of SOD, CAT, and GPx [108].

Nrf2 tightly regulates the transcriptional machinery for important cellular defense systems including antioxidative and phase II drug-metabolizing enzymes. Physiologically Nrf2 remains bound to Keap1 as an inactive complex. Chemical or electrophilic modification of Keap1 via ROS renders Nrf2 free for its translocation to the nucleus. In the nucleus, Nrf2 activates the antioxidant-responsive element and the electrophilic-responsive element, stimulating expression of relevant transcriptional proteins. Nrf2 is reported to be involved in cellular adaptive responses against As<sub>2</sub>-induced oxidative stress [153,154]. Arsenic-induced Nrf2 activation has been reported in various cell lines including MC-3T3E osteoblast cells and HaCaT, resulting in up-regulation of HO-1, Prx1, A170,

NAD(P)H-quinone oxidoreductase 1, and  $\gamma$ GCS. Whereas acute arsenic exposure (10  $\mu$ M, 2–6 h) resulted in Nrf2 activation, mediated through oxidative stress, the reverse may be true in the case of chronic arsenic exposure (100 nM, 28 days). It is speculated that this may be due to a high level of oxidative stress. It is also reported that arsenic-induced malignant transformation of the HaCaT cell line could be attributed to elevated basal Nrf2 activity and decreased Nrf2-mediated antioxidant response. This paradox was attributed to negative feedback from Nrf2-mediated increased GSH biosynthesis, or increased activity of Nrf2 downstream phase II enzyme causing arsenic detoxification, or increased Nrf2 degradation via CK-2 activation in tumor cells [50,153–156].

### Biomarkers of arsenic-induced oxidative stress

As discussed above, it is very evident that the effects of arsenic are mostly ubiquitous. It is thus imperative to look at various markers to study or validate arsenic exposure. The signs and symptoms of arsenic exposure depend on the extent, degree, and duration of exposure. Diagnosis and assessment of the extent of arsenic toxicity can be carried out by measuring defined markers indicative of either biological injury or the presence of arsenic in the biological environment. To achieve the latter, measuring arsenic levels using atomic absorption spectroscopy in various biological samples such as urine, blood, or hard tissues such as nails and hair might be a convenient solution. However, this may not be true for cellular studies. Further, correlation of arsenic toxicity with the extent of injury is rather a challenge. Looking at it from a different prospective, identifying easy early diagnostic parameters in a population not known to be exposed to arsenic is rather a necessity to proceed further with metal estimation in patient samples.

In this section I discuss biomarkers that may be of significance in estimating arsenic-induced oxidative damage. This will further facilitate the assessment of the body's antioxidant status, which may determine the appropriate clinical intervention.

### Reactive oxygen species

One of the earliest responses to arsenic toxicity is the increase in ROS levels in blood and soft tissues. Techniques using fluorescent probes and electron spin resonance (ESR) have made it possible to estimate ROS in biological samples, allowing their use as an early indicator of oxidative stress. However, employing ROS as a potential diagnostic marker remains a challenge with methodological limitations. A number of fluorescent probes are commercially available that can detect various radicals ( $^1\text{O}_2$ ,  $\text{H}_2\text{O}_2$ ,  $^{\bullet}\text{OH}$ , or  $\text{O}_2^{\bullet-}$ ), an example being 2',7'-dichlorodihydrofluorescein diacetate [71]. Despite extensive use these share limitations such as high sensitivity yet nonspecificity and photo-instability that restrict their candidacy for human use [157]. Other techniques such as ESR that can detect specific free radicals such as  $\text{O}_2^{\bullet-}$  [31] and  $^{\bullet}\text{OH}$  are also known. Because unpaired electrons are short lived, employing spin traps such as Tempol-H to ESR spectroscopy further enhances results [158]. Immunodetection of spin trapping agents could be another potential approach to studying radicals; however, these techniques have rarely been used in human samples of arsenic toxicity [71,159,160].

On the other hand, endogenous generation of ROS as a by-product of normal metabolism as well as arsenic exposure further restricts their use as an early arsenic toxicity biomarker. ROS-induced damage to important biomolecules such as lipids, proteins, and DNA has also been used as a biological indicator for arsenic-induced oxidative stress.

### DNA oxidation products

Arsenic-induced DNA damage mediated through ROS has been linked to carcinogenesis/mutagenesis [2]. The presence of unsaturated bonds in purine and pyrimidine rings makes DNA sensitive to free radicals. Arsenic degrades DNA either by generating free radicals or by causing malfunctions in DNA-repair enzymes or both. Among DNA bases, guanine is the most sensitive nucleotide for free radicals, and after oxidation it becomes converted into various markers such as 8-hydroxyguanine, 8-hydroxyguanosine, and 8-hydroxy-2-deoxyguanosine (8-OHdG) [71,161]. 8-Oxoadenine, the oxidized form of adenine, has also been detected in urine of arsenic-exposed animals [162]. Expression of 8-oxoguanine-DNA glycosylase, a DNA damage marker that removes 8-oxoguanine from DNA, has been correlated with both the repair capacity of 8-oxoguanine [163] and contaminated water and nail arsenic concentrations in exposed subjects [164]. Analytical methods utilized to detect these markers are technically challenging as they become transformed into stable end products [26]. Moreover, the artifactual adventitious oxidation generated during DNA extraction and sample preparation results in high background reading that may exceed arsenic-induced increases in DNA markers. Employing immunospin trapping as in the ODD test could make the detection of arsenic-induced DNA damage more dependable [53].

Arsenic is also reported to interfere with the DNA repair machinery by either inhibiting ligation and incision steps or down-regulating the gene expression of DNA repair enzymes such as excision repair cross-complement 1 in humans [71]. The single-cell gel electrophoresis or comet assay has been extensively used for this purpose despite limitations [162–165]. Thus, other more specific DNA repair markers from base or nucleotide excision DNA repair pathways are being identified [71].

### Lipid peroxidation

The role of lipid peroxidation in As-induced oxidative stress has been extensively studied. A positive correlation between plasma lipid peroxidation and arsenic levels in urine and/or tissue samples has been documented in both clinical and preclinical studies [71,166,167]. Arsenic-induced ROS directly attack the hydrogen atom of a methylene group adjacent to an unsaturated carbon atom.

Polyunsaturated fatty acids are more sensitive to free radical damage and form malondialdehyde (MDA), HNE and 2-propenal (acrolein), and isoprostanes, which can be measured in plasma and urine as indirect indicators of oxidative stress [130,132,167]. These peroxidized products of lipid can be quantified biochemically, through a thiobarbituric acid test; however, the test has some limitations [168].

### Protein oxidation

Free radical-mediated damage to proteins may be initiated by electron leakage during metal-ion-dependent reactions and autooxidation of lipids and sugar. Arsenic-induced ROS such as  $\cdot\text{OH}$  or  $\text{O}_2^{\cdot-}$  are the main cause of oxidative damage to proteins [141]. A known biomarker generally used to estimate protein oxidation is protein carbonyls. Arsenic-induced protein carbonyl content can be detected using dinitrophenylhydrazine-based calorimetric methods or ELISA [169,170].

There is a significant correlation between arsenic exposure and protein oxidation; the latter is relatively less studied compared to other variables. Clinical as well preclinical reports have suggested utilization of protein carbonyl content as an arsenic-induced oxidative stress marker [171,172]. Recently, high-end techniques such as the 2,4-dinitrophenyl hydrazine assay, two-dimensional gel electropho-

resis, and MALDI-TOF-MS have been utilized to estimate arsenic-induced protein damage [173].

### Other biological indicators

Toxic metals have a strong affinity for nucleophilic ligands; therefore, each step of the heme biosynthesis pathway is susceptible to direct inhibition via metal-mercaptide bond formation with the functional sulfhydryl groups [174]. Arsenic exposure produces dose-related increases in urinary excretion of uroporphyrin and coproporphyrin [175].  $\delta$ -Aminolevulinic acid dehydratase (ALAD), an enzyme that catalyzes the asymmetric condensation of two molecules of ALA to porphobilinogen, is highly sensitive to the presence of arsenic by virtue of its sulfhydryl moiety [142]. Subchronic exposure increases hepatic  $\delta$ -aminolevulinic acid synthetase activity in rats, interpreted as a reduction in the hepatic free heme pool due to induction of heme oxygenase [176]. Human lymphocyte heme oxygenase has been proposed as a biomarker of response to environmental arsenic exposure [177].

### Antioxidant levels

Antioxidant defense is one of the most efficient mechanisms protecting biological molecules, but also a major target for toxic compounds. Thus, altered levels of these antioxidants provide insight into the degree, extent, or stage of arsenic-induced oxidative damage, serving as biomarkers of arsenic toxicity. The antioxidant activity of enzymes such as SOD, CAT, and GPx is directly or inversely proportional to arsenic toxicity depending upon the dose and time of arsenic exposure. GST has been proposed a biomarker for arsenic toxicity after evaluating all pulmonary biomarker-based alterations [178].

### Pathophysiology of arsenic-induced ROS-mediated diseases

#### Hepatic and renal disorders

The association between arsenic exposure and human liver cancers has been mixed [179–184]. A positive correlation between chronic arsenic exposure in humans and hepatotoxicity attributed to oxidative stress has also been reported [185]. In a mouse model, chronic arsenic exposure affected the antioxidant defense enzyme levels and ROS-mediated lipid peroxidation preceding pathomorphological lesions and hepatic fibrosis [149]. Liver fibrosis initiates with capillarization that further progresses with remodeling and shunting. Arsenic exposure activates the NADPH oxidase 2 (Nox2)-based oxidase in sinusoidal endothelial cells, which plays a central role in arsenic-induced capillarization. It has been suggested that Rac1 and oxidase activation are downstream of the target of arsenic in endothelial cells because Rac1-GTPase activity was found essential for capillarization. Further, arsenic signaling in sinusoidal endothelial cells possibly ends with the formation of peroxynitrite [186]. Elevated hepatic enzymes, jaundice, and hepatic fibrosis that cause portal hypertension but do not progress to cirrhosis have been characteristic of chronic arsenic toxicity in West Bengal [187,188]. Fetal arsenic exposure may lead to tumor formation in adulthood in various organs, including the liver [188].

Arsenic-induced hepatotoxicity may follow multiple mechanisms, including ROS-mediated oxidative stress, inflammatory response, or metabolic hindrance. Acute high-dose arsenic exposure up-regulates a number of oxidative stress-related genes (heme oxygenase 1 and metallothionein) [189] that were not observed after low-dose, chronic arsenic exposure [190]. Moreover, we reported that subacute and chronic arsenic exposure in vivo resulted in ROS generation, decreased GSH/GSSG ratio and antioxidant enzymes, and increased lipid peroxidation in hepatic tissues [191–194]. Arsenic-induced

hepatocarcinogenesis has been associated with an increase in the oxidative DNA damage biomarker 8-OHdG [179,195,196]. Prolonged arsenic exposure caused a dose-dependent increase in collagen accumulation correlated with elevated interleukin-6 (IL-6) concentrations [197]. PDH, an important enzyme of glucose metabolism, is susceptible to arsenic-induced ROS generation [198]. Inhibition of this enzyme may cause disorders associated with carbohydrate metabolism such as diabetes [199].

Liver, being the prime site of arsenic metabolism, is subject to close interaction with various arsenic species. MMA<sup>III</sup> shows the greatest binding with hepatocytes, followed by arsenite, with 60% of the MMA<sup>III</sup>-binding activity, and DMA<sup>III</sup>, arsenate, MMA<sup>V</sup>, and DMA<sup>V</sup>, each showing about 15% of the binding [200]. During arsenic metabolism in liver, conversion of As<sup>3+</sup> to As<sup>5+</sup> under physiological conditions leads to ROS generation. Injury to hepatocyte mitochondria suggests mitochondria to be one of the prime targets of arsenic-induced ROS [201].

Similar to liver, the kidney is another important target organ of arsenic toxicity because it is involved in arsenic toxicokinetics such as absorption and accumulation. The highest concentrations of arsenic can be detected in kidneys and liver after acute arsenic exposure [202]. Arsenic-induced oxidative stress in hepatic and renal microsomes damaged cellular lipids and proteins and caused a decrease in antioxidant and xenobiotic metabolizing enzyme activity in rats [203]. Epidemiological studies in various countries such as Argentina [204], Chile [205], Japan [206], Taiwan [17], and the United States [207] suggested an increased risk of urinary cancer due to arsenic-contaminated drinking water. Arsenic-induced ROS are one of the important mechanisms of DNA damage that leads to mutagenesis and/or carcinogenesis [208,209]. Arsenic is capable of causing acute renal failure, as well as chronic renal insufficiency. The actual cause of injury may be hypotensive shock, hemoglobinuric or myoglobinuric tubular injury, or the direct effect of arsenic on tubule cells, because kidneys are the main route of arsenic excretion. Sublethal arsenic poisoning has resulted in renal insufficiency and necrosis [210]. The fundamental lesions in arsenic toxicity are the loss of capillary integrity and increased glomerular capillary permeability, which may result in proteinuria. Other renal injuries were diagnosed by the presence of hematuria, leukocyturia, and glycosuria. In some cases of acute and subchronic arsenic exposure, elevated serum levels of creatinine have been noticed [211]. Renal failure has also been reported rarely [212]. Renal functional impairment is mainly seen in acute poisoning by arsenic, with oliguria and anuria progressing to renal failure and sequelae including chronic renal insufficiency and hypertension.

### Cardiovascular disorders

Cardiovascular diseases such as hypertension, QT prolongation, peripheral arterial disease, atherosclerosis, impaired microcirculation, coronary heart disease, and stroke are well recognized in relation to dose and duration of arsenic exposure [213,214]. These manifestations may be classified as cardiac and vascular diseases [214]. Reports have also demonstrated a positive correlation between mortality due to ischemia and arsenic exposure [215,216]. A direct effect of arsenic on cardiac muscles may be characterized by the electrophysiological abnormalities reported in subjects with prolonged arsenic exposure. A study of 280 females and 350 males in an arsenic-endemic area in Taiwan showed significant correlation between arsenic dose and QT prolongation and increased QT dispersion [217]. The reduced nerve conduction amplitude too followed a dose-response relationship [218]. The latter may be of relevance to previous reports of arrhythmias and pericarditis as arsenic-induced cardiotoxic effects [219–221]. Thus corrected QT-interval prolongation may be a potential early diagnostic marker for ischemic heart disease or carotid atherosclerosis in arsenic-exposed populations [220].

Arsenic induces cytotoxic effects in cardiomyocytes that are mediated through ROS leading to apoptosis via caspase-3 signaling. These cytotoxic effects also have cellular manifestations *in vitro* such as loss of cardiac actin, reduced size, and damage to the nuclei, which coordinate well with disruption of the vascular extracellular matrix *in vivo*. It is also suggested that arsenic-induced cardiomyocyte toxicity *in vitro* is concentration dependent, with an EC<sub>50</sub> of about 1 mM [222,223]. It is interesting to note that treatment of patients with arrhythmias with verapamil, an antihypertensive and the most widely used calcium antagonist, may accentuate the effects of arsenic in a dose-dependent manner. Thus, patients receiving this drug may be at a higher risk of cardiac complications from arsenate toxicity [222].

Risk of endemic peripheral and systemic vascular disease has been associated with chronic arsenic exposure. The first few reports establishing a dose-response relationship between the extent of arsenic exposure and blackfoot disease goes back to 1977 [224]. Chronic, high arsenic exposure is associated with prevalence of obesity, hypertension, and other cardiovascular disease where genetic variations distinguish this prevalence. Subjects with decreased arsenic methylation and excretion efficiency and who are TNF $\alpha$ -308A allele and IL-6-174 G carriers were more susceptible to arsenic-induced cardiotoxicity [225]. Two risk genotypes of ApoE and monocyte chemoattractant protein 1 (MCP1), which are related to inflammatory response, in humans have been recently identified. These subjects, when exposed to >10  $\mu$ g/L, have shown >10-fold risk of carotid atherosclerosis [214,226]. The roles of proinflammatory chemokines (MCP1), cytokines (IL-6), oxidative stress markers, and protein-MDA adducts have also been reported in arsenic-exposed experimental animals [227]. A recent study using pregnant ApoE-knockout (ApoE<sup>-/-</sup>) mice concluded that *in utero* arsenic exposure induces an early onset of atherosclerosis measured as accelerated development of aortic lesions and vasorelaxation defects without a hyperlipidemic diet [228]. Such early life exposure to arsenic opens a new discipline of understanding that is beyond the purview of this review [229,230].

Atherosclerosis is a multifactorial pathophysiological process of arterial vasculature. Arsenic has been shown to have a dose- and time-dependent inhibitory effect on proliferation and viability of endothelial cells and may cause endothelium dysfunction leading to apoptosis via the induction of p21 [231,232]. Moreover, arsenic-induced oxidative stress can also suppress Fas ligand expression, leading to endothelial dysfunction [233]. As discussed earlier in cellular defence mechanisms (Section 9) arsenic may stimulate NAD(P)H oxidase-generating superoxide [133]. This superoxide may deplete NO levels and modulate the vascular tone. Reports have also demonstrated that arsenic and MMA<sup>III</sup> may also directly reduce the bioavailability of NO by inhibiting nitric oxide synthase (NOS) activity [234,235] (Section 8).

Oxidized low-density lipoproteins (LDLs) function to modulate intracellular signal transduction in coordination with up-regulation of inflammatory mediators and adhesion molecule gene expression. These LDLs, present in all stages of atherogenesis, generate a number of bioactive molecules (ROS, peroxides, and isoprostanes), most abundantly HNE. Increased accumulation of protein adducts of MDA and HNE in arsenic-exposed mouse during both early and advanced vascular lesions thus indicates the role of LDL [214].

Arsenic-induced initiation of atherosclerosis may be summarized as follows: (1) Arsenic presents an oxidative insult to the vascular cells, especially the endothelium, resulting in its activation and release of inflammatory chemical mediators, adhesion molecules, and chemokines. (2) These attract the platelets and monocytes that cause further activation, initiating a vicious circle. (3) The oxidized LDL, when scavenged by macrophages, results in foam cell formation (macrophages loaded with lipids). (4) Reduced NO bioavailability contributes to vasoconstriction and increased transcription factors such as NF- $\kappa$ B, which further stimulate inflammatory processes. Moreover, arsenic through peroxynitrite formation and NF- $\kappa$ B

activation increases expression of cyclooxygenase-2 in endothelial cells [236,237]. (5) Finally, arsenic inhibits tissue-type plasminogen activator and promotes plasminogen activator inhibitor type 1, thus reducing fibrinolysis, which worsens the scenario and promotes coagulation processes [238]. The progression of atherosclerosis continues until the blood vessel is obliterated by plaque formation [239,240].

Hypertension is reported to be elevated in arsenic-exposed subjects [183,241]. Although only few epidemiological studies have reported the dose–response relationship between arsenic ingestion and hypertension [242,243], increased hypertension prevalence among patients with arsenic-affected skin lesions has been previously known [244,245]. The mechanism underlying arsenic-induced hypertension closely resembles that explained for atherosclerosis, in which vascular redox signaling is suggested to be mainly involved. However, specific mechanistic details are unavailable because of the lack of an ideal experimental animal model [246]. However, several factors have been identified. Nox enzyme complexes, as discussed, may play a central role [214] and this induction requires Rac1- or Cdc42-GTPase activity along with the involvement of type-1 sphingosine-1-phosphate receptor [247]. Also reduced NO bioavailability may be a major factor involved in the arsenic-induced hypertension [248]. Other mechanisms may include alteration of blood pressure regulating pathways, arsenic-induced hepatic, and renal and neurological defects [214,249].

#### Type 2 diabetes

The role of arsenic in type 2 diabetes is currently controversial. Whereas epidemiological studies in Bangladesh and Taiwan show a dose-dependent correlation, studies in the United States on low or occupational exposures show no correlation [183,250–256]. The reasons for such controversy could be many, starting with misleading parameters such as questionnaires, limited tests performed, confusing inclusion or exclusion criteria, and unclear end points such as mortality and morbidity [257].

Although the basic mechanism underlying the pathophysiology of arsenic-induced diabetes has been unclear, the theoretical understanding and experimental data clearly indicate the possible targets. Epidemiological data conclude that arsenic-induced symptoms mimic a type 2 diabetes-like syndrome, which is characterized by a dual mechanism of insulin resistance and reduced insulin secretion in arsenic-exposed populations [258]. Type 2 diabetes initiates with an undetected insulin resistance that is compensated for by hyperinsulinemia, slowly progressing to pancreatic  $\beta$ -cell injury, dysfunction, or death [258,259]. Glucose metabolism itself leads to generation of ROS that serves as an important signal for glucose-stimulated insulin secretion. In the process Nrf2 mediates cellular adaptive responses to oxidative stress. Low-level arsenic exposure in vitro hampers  $\beta$ -cell functioning by activating the adaptive cellular response to oxidative stress involving Nrf2 by reducing the glucose-stimulated insulin secretion in a time- and dose-dependent manner [260]. Moreover, subchronic low-level (0.5–2  $\mu$ M) arsenic exposure reduces the free  $[Ca^{2+}]_i$  oscillations important for glucose-stimulated insulin secretion, thus reducing calcium-dependent calpain-10 partial proteolysis of SNAP-25. The calcium–calpain pathway triggers the exocytosis of insulin in RINm5F cells. Arsenic also hampers  $\beta$ -cell proliferation by causing arrest at G2/M, possibly through ROS as a secondary messenger [259]. Arsenic inhibits insulin release; this does not modulate or reverse arsenic-induced diabetes in vivo. Diabetes modulates the pharmacodynamic and pharmacokinetic fate of arsenic in rats resulting in reduced hepatotoxicity possibly due to non-insulin-dependent carbohydrate regulatory metabolism [261].

Sulfhydryl groups, structurally and functionally, play a crucial role in insulin and other pathways mediating glucose transport. The thiol moiety is an important target for arsenic because it is a structural link (disulfide bond) between the A and the B polypeptide chains of

insulin, the  $\alpha$  and the  $\beta$  subunits of the insulin receptor, and the exofacial sulfhydryl groups present on GLUTs at the cell membrane. It may be noteworthy that glucose transport impairments in skeletal and adipose tissues play a leading role in the pathogenesis of type 2 diabetes. Thus, sulfhydryl groups may be crucial targets, but a very high concentration of arsenic is required for the effect, which may not correlate with concentrations found in vivo [262].

Arsenic-induced oxidative stress can directly contribute to  $\beta$ -cell dysfunction and indirectly cause insulin resistance via stress-sensitive pathways that involve transcription factors such as NF- $\kappa$ B. Pancreatic  $\beta$  cells are highly susceptible to oxidative insult, with a natural deficiency of antioxidant defense enzymes such as GPx, CAT, and SOD [263]. ROS induce the formation of islet amyloids in pancreatic cells by rapid polymerization that accumulates to cause lesions, thus hampering insulin release and ultimately leading to islet cell injury and death. Loss of  $\beta$  cells by progressive amyloidosis is a characteristic pathological finding with type 2 diabetes [264]. In the absence of sufficient experimental results we may hypothesize that arsenic via ROS generation may cause structural damage resulting in the dysfunction or death of pancreatic  $\beta$  cells. Low arsenic concentrations induce NF- $\kappa$ B gene expression (see Section 5) that regulates expression of cytokines such as TNF $\alpha$  and IL-6 that are well documented to play a part in the mechanism of insulin resistance in adipose tissue [265,266]. Arsenic-induced up-regulation of TNF $\alpha$  may increase the serine phosphorylation of insulin receptor substrates, thus reducing their ability to dock with the receptor and their interaction with downstream events [255]. Chronic in vitro exposure of adipocytes to IL-6 has shown to diminish glucose transport by down-regulating the expression of the insulin receptor  $\beta$  subunit and GLUT4 [267]. Arsenic also inhibits the adipose-selective nuclear receptor PPAR $\gamma$  (peroxisome proliferator-activated receptor  $\gamma$ ), which plays an important role in insulin-dependent glucose homeostasis. Whereas PPAR $\gamma$  activation decreases TNF $\alpha$ , IL-6 treatment down-regulates PPAR $\gamma$  in adipocytes [267]. Thus, arsenic-induced insulin resistance may be directly or indirectly mediated via ROS generation, which modulates expression of NF- $\kappa$ B, which regulates cytokines and interacts with PPAR $\gamma$ . Arsenic modulates the expression of various genes that may cause decreased insulin secretion, induced peripheral insulin resistance, alterations in gluconeogenesis, induced oxidative stress in and decreased proliferation of pancreatic  $\beta$  cells, and changes in the proliferation and differentiation of muscle cells and adipocytes [258].

#### Neurological defects

Arsenic-induced neurological defects have been known more as isolated case reports than detailed epidemiological studies [268,269]. Depending on the dose, duration, and route of arsenic exposure, neurological dysfunctions can range from neurobehavioral disturbances to memory and cognitive impairments, visual or auditory sensory defects, and peripheral neuropathies and encephalopathies. However, the cellular and molecular mechanisms of arsenic-induced neurological defects are not clearly defined.

Most common symptoms reported after chronic arsenic exposure include numbness of distal extremities, especially the legs; decreased sensibility, ataxia, pain and paresis with nausea and vomiting, indicative of systemic peripheral neuropathies [270]. Despite numerous reports, the mechanism for arsenic-induced peripheral neuropathies is yet to be elucidated [271–274]. In arsenic-induced neuropathy, the longer axons of sensory neurons are more affected than motor neurons. Demyelination or cytoskeletal defects in neuronal axons are the commonly known pathologies for peripheral neuropathy that hampers the conduction velocity of peripheral nerves, as demonstrated in smelter workers exposed to 50  $\mu$ g arsenic/m<sup>3</sup>. Histopathological reports of patients with arsenic-induced neuropathy documented fragmentation and resorption of myelin, disintegration of axis cylinders, and depleted

myelinated fibers [275,276]. Such reports are supported by *in vitro* and *in vivo* preclinical studies showing that arsenic (10  $\mu$ M) inhibits myelination and decreases neurite growth by 50% in a dorsal root ganglia culture [275]. Arsenic-induced demyelination may be due to the decreased methylation of basic myelin protein at the arginine residue through protein methylase I, which is a crucial posttranscriptional process contributing to the integrity of myelin [277]. Arsenic possibly interferes with other cellular methylation pathways, contributing to the neurotoxicity. Arsenic-exposed rats experienced electrophysiological changes, which were consistent with histological alterations showing demyelination and increased lipid peroxidation in peripheral nerves. These manifestations adversely affected the generation and propagation of action potentials in peripheral nerves and reduced the transmission from peripheral sensory organ to the central nervous system [278].

The role of cytoskeletal defects in arsenic-induced peripheral neuropathies has been recently highlighted. Nerve cytoskeletal proteins are a flexible framework for cells that enables communication between cell parts along with other functions. Neurofilaments (NF-H, NF-M, NF-L), cytoskeletal proteins, are the major components of large myelinated neurons. Within peripheral nerves, arsenic causes a reduction in NF-L, which is the only NF protein that is capable of independently organizing and coassembling *in vivo*. NF-L is crucial and is required by both NF-H and NF-M to form a heteropolymer in the cytoskeleton of the neuronal cell. Arsenic possibly binds covalently with the -SH moiety of NF components, especially NF-L, and induces degradation. Thus arsenic concentration in peripheral nerves is inversely related to NF-L levels, because arsenic-induced degradation of NF-L also leads to arsenic losing its binding site, thus reaching the circulation again. Arsenic-induced NF-L degradation has also been attributed to either increased content of calpains and subsequent  $\text{Ca}^{2+}$ -induced proteolytic process or increased activity of calcineurin, a calcium-dependent serine/threonine phosphatase [279,280]. Sodium arsenite decreases the NF transport into axonal neuritis and increases perikaryal phosphor-neurofilament immunoreactivity. The latter may be mediated through JNK and not cyclin-dependent kinase 5 and p38 MAPK [281]. As discussed previously, JNK belongs to the MAPK family, which is responsible for handling stress that, in the case of arsenic, may have been caused by ROS generation [282,283]. Thus, arsenic targets NFs by interfering with the posttranscriptional processes by disturbing the methylation pathways, stimulating phosphorylation, or inducing NF degradation by calcium-dependent cytoplasmic protease [284]. Also as a secondary messenger in cell signaling, the role of arsenic-induced ROS in support of the proposed hypothesis may not be ignored. Conclusively arsenic perturbs the neurofilament dynamics, possibly with the involvement of oxidative stress that contributes to the development of peripheral neuropathy.

Arsenic-induced brain encephalopathy reports are indicative of central nervous system disruption [285]. The severity and reversibility of arsenic-induced CNS manifestations are related to exposure duration and may disappear with the cessation of exposure in some cases while being irreversible in others [269,286]. Other manifestations including sensory impairment, such as visual and auditory, and memory and cognitive defects have also been reported in clinical cases of arsenic toxicity [287].

The role of oxidative stress as the leading mechanism in arsenic-induced neurological defects has been widely supported by *in vitro* and *in vivo* studies. In occupationally arsenic-exposed subjects a positive correlation between compromised subjective neurological symptoms, visual evoke potential, electroneurographic and electroencephalographic results, and arsenic concentration in air and urine was established. Oxidative stress may be the initiating mechanism for arsenic-induced neurotoxicity [288]. Arsenic-induced DNA damage and apoptosis in neuronal cells may follow an intrinsic mitochondrial apoptotic pathway, mediating through increased intracellular calcium that triggers mitochondrial stress and generation of ROS [284].

Further, chronic arsenic exposure inhibits the cholinergic system, decreasing acetylcholine synthesis, release, and reuptake. A decrease in AChE activity in cerebellum, hypothalamus, and brain-stem homogenates in rats subjected to 2–4 months arsenic exposure in drinking water has been shown [275]. The central effects of arsenic may also be correlated with the ability of arsenic to modulate the kinetics of essential elements in brain. Arsenic after ingestion follows linear kinetics for its accumulation in the brain until it reaches saturation. A study reports a uniform distribution of arsenic in mouse brain and its ability to redistribute essential elements such as copper, zinc, and iron as a possible mechanism for its neurotoxic effects [289].

### Carcinogenesis

Epidemiological studies indicate that populations exposed to arsenic are at a high risk of developing skin, bladder, liver, and lung cancers [290–295]. Arsenic is a class 1 carcinogen, but the detailed etiology and risk assessment for establishing permissible exposure limits remain controversial [296]. This can be attributed to various factors such as (i) arsenic species exhibiting varying degrees of toxicity, (ii) unavailability of the right biological screening model that may mimic human exposure, or (iii) conflict of decision on appropriate risk assessment model for arsenic-induced cancer in humans [297–300]. Moreover, the mechanism for arsenic-induced carcinogenesis is yet to be understood [301].

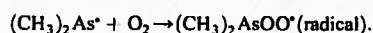
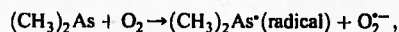
Arsenic compounds are classified as human carcinogens and *in contrast* have also been used as therapeutic agents to treat acute promyelocytic leukemia. We may hypothesize that arsenic, under some circumstances, may have cytotoxic effects via necrosis or apoptosis, whereas in other situations arsenic pushes the cell away from apoptosis to undergo uncontrolled/defective proliferation. Owing to its speciation and dual effects, arsenic may exhibit more than one mechanism toward the exposed cell. These mechanisms may include oxidative stress via ROS and RNS generation, protein binding, DNA damage or repair process impairment, gene expression modulation, and interfering with cytoplasmic and nuclear signal transduction pathways that may be mutually dependent on or independent of each other.

Many *in vitro* and *in vivo* studies have reported the pro-oxidant status of arsenic [302]. Arsenic-induced oxidative stress stimulates defense mechanisms that counter stress by scavenging free radicals and repair the damaged cellular biomolecules. This is attempted by activating various pathways including expression of antiapoptotic, and inhibition of proapoptotic genes for which ROS act as secondary messengers [303]. When oxidative stress overwhelms the cellular defenses and macromolecular damage is irreversible, the cell undergoes apoptosis. Thus, it is the duration and extent of free radical exposure that decide the fate of cells subjected to such stress [304,305]. Moreover, several examples discussed in previous sections confirm that although low arsenic concentrations activate certain processes or pathways, its high concentration inhibits the same (e.g., NF- $\kappa$ B, angiogenesis).

The arsenic–protein binding theory of carcinogenesis has been rather recently established. The most important protein targets include PARP-1 and xeroderma pigmentosum protein A (XPA; both DNA repair proteins) and tubulin. This theory describes events that initiate with arsenic-SH binding at various enzymatic sites followed by downstream events that include enzymes that alter signal transcription and cellular redox status (thioredoxin and glutathione reductase), cause DNA methylation and impaired DNA repair, and induce chromosomal aberrations (tubulin). Arsenic is reported to cause DNA modifications such as aneuploidy, micronuclei formation, chromosomal aberrations, deletion mutations, sister-chromatid exchange, and DNA–protein cross-linking, which may be due to tubulin protein binding [306]. Moreover, as discussed earlier, arsenic targets DNA repair enzymes such as PARP, XPA, etc., via ZF inhibition

[138,139,307]. The above-described pathways together result in mutation accumulation typically in oncogenes or tumor suppressor genes, ending in high risk of cancer [138].

Arsenic-induced oxidative stress has been a more accepted hypothesis for carcinogenesis [299]. The major determinant of arsenic-induced oxidative stress-mediated DNA damage remains the valence state of the arsenic species. Intermediate arsenic metabolites form free radicals that directly interact with cellular macromolecules. For example:



The genotoxicity of trivalent arsenic species is higher compared to its pentavalent counterpart as discussed in Section 3 on arsenic methylation, owing to their higher ability to release iron from ferritin. This allows iron to catalyze the formation of hydroxyl radical from  $\text{H}_2\text{O}_2$  available for DNA damage [308]. Because arsenic causes DNA modifications via ROS attack, it fails to cause point mutations characteristic of any classical mutagen. Nitric oxide synthase inhibitors,  $\text{O}_2^{\bullet-}$  scavengers, and peroxynitrite scavengers reduce arsenic-induced DNA strand break in aortic cells, confirming the role of ROS and RNS production in the process. Among ROS, hydroxyl radical is believed to be the major reactant species that interacts with all four DNA bases [306]. Further, the DNA repair mechanism is inhibited by arsenic either directly or indirectly by altering cellular redox levels, affecting signal transduction pathways or phosphorylation of proteins by the enzyme DNA ligase [31]. Thus, such cellular interactions of arsenic enhance proto-oncogenes and/or inhibit tumor suppression genes in the absence of DNA repair, and the cell is likely to become cancerous.

Whereas DNA modification or damage may cause tumor initiation, arsenic-induced ROS may support tumor promotion by acting as secondary messengers to modify the cell transformation response, cell signaling pathways, gene expression, and transcription factors. Arsenic is known to differentially affect the transcription factors that participate in activating or inhibiting the process of cell proliferation. In reference to section on arsenic-induced altered mitochondrial and enzyme activity (Sections 7 and 8), Sections 7 and 8, this section focuses only on arsenic-induced alterations in signal transduction and transcription factors during carcinogenesis. The elevated levels of tyrosine phosphorylation as induced by arsenic have been associated with abnormal cell signaling and abnormal cell growth leading to development of cancers including prostate, lung, bladder, kidney, colon, and skin cancers [80,309–313]. Similarly, activation of the MAPK family shows uncontrolled proliferation upon ERK induction and apoptosis after JNK induction. Arsenic-transformed cells exhibited less activity than nontransformed cells, whereas the ERK and p38 MAPKs were similar in both cells [195]. NF- $\kappa$ B activation as described for low-level arsenic is associated with turning-on of numerous antiapoptotic genes and induction of proliferation and initiation or acceleration of tumorigenesis [314,315].

The Hedgehog pathway, identified as facilitating maintenance and progression of various tumors, has also been found to be activated by arsenic exposure in various *in vitro* and *in vivo* models. Arsenic reduces the stability of GLI3, a Hedgehog-regulating transcription factor. The authors reported a positive correlation between Hedgehog activity and arsenic exposure in tumor samples from bladder cancer patients [316]. Arsenic was found to interact with tumor suppressor genes such as p53 indirectly by up-regulating the expression of its regulators, such as MDM2 protein [306]. Thus the cell may progress to division and the damaged DNA (due to arsenic) replicates because of suppressed p53 expression along with DNA ligase inhibition. The arsenic-induced p53 expression response may be complex and dose, duration, cell, and tissue-type specific [62]. The cell cycle checkpoints are stimulated by *in vitro* arsenic treatment of cells. The effect is

suggested to be differentially mediated by MAPK member kinases and NF- $\kappa$ B. Inhibition of NF- $\kappa$ B by high arsenic concentrations increases the cell cycle inhibitory protein GADD45, thus facilitating apoptosis. However, arsenic at low concentrations may act as a carcinogen by activating NF- $\kappa$ B in normal cells by preventing cell cycle checkpoint activation. In contrast, JNK inhibition causes decreased arsenic-induced GADD45 activation, further suggesting its role in the anticarcinogenic effect of arsenic [306,315].

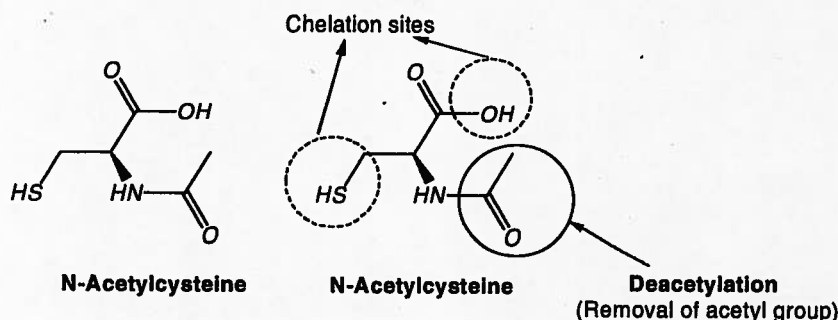
In humans, arsenic is reported to cause cancers of lung, bladder, and skin. This may be evidently explained on the basis of the oxidative stress theory of arsenic-induced cancer. All these tissues exhibit high arsenic turnover. Arsenic remaining after urinary elimination is suggested to be deposited in lung, liver, and keratinized tissues such as skin, nail, and hair. DMA, being a gas, is eliminated through the pulmonary tissue, where intermediate radical formation may be higher because of high oxygen partial pressure. Further, in cases of inhalational exposure, arsenic particles may undergo a much prolonged kinetic, thus being retained in the tissue, eliciting oxidative and inflammatory responses [292]. Similarly, the bladder lumen retains high concentrations of DMA and MMA and their trivalent species ( $\text{DMA}^{\text{III}}$  and  $\text{MMA}^{\text{III}}$ ), formed after reduction. High sensitivity of liver and renal tissue is also by virtue of arsenic metabolism and excretion, which provide a close proximity of their cells to arsenic intermediates. Keratinized tissues such as skin, on the other hand, are susceptible because of high arsenic deposition in these tissues. The most likely human arsenic carcinogens include arsenites,  $\text{MMA}^{\text{III}}$  and  $\text{DMA}^{\text{III}}$  [18].

Arsenic has also been reported to potentiate the carcinogenic potential of other carcinogens under both *in vitro* and *in vivo* experimental conditions. It shows synergistic mutagenic effects with UV light or carcinogenic chemicals when mammalian cells or mice are coexposed [317]. The DNA ligase inhibition potential of arsenic is seen as a contributing mechanism of such synergism [318]. These preclinical reports are supported by epidemiological studies from Mexico, Taiwan, and Japan. In populations exposed to arsenic, a higher incidence of skin and lung cancer is shown and believed to be caused by UV radiation and chemical carcinogens from cigarettes, betel nuts, and other substances [302,306,319].

Vascular malignancies such as primary hepatic angiosarcoma and hemangioendothelial sarcoma have been documented with arsenic exposure. Angiogenesis is an essential process in the development of neoplastic disease. Arsenic at low concentrations stimulates or facilitates angiogenesis *in vitro* via the VEGF-NOS-NO signaling pathway; however, higher arsenic concentrations ( $>5 \mu\text{M}$ ) inhibit angiogenic morphogenesis. The role of endothelial NOS in VEGF-induced angiogenesis via NO activity has been suggested in the process. Increased NO production by arsenic could involve a calcium-dependent pathway because NOS3 was up-regulated and not NOS2. This study further confirmed that, whereas submicromolar arsenic concentrations prime the cells for amplified mitogenic response, higher concentrations result in a cytotoxic response interrupting with cell cycle [320].

#### Use of antioxidants (synthetic or herbal)/chelating agents in reducing arsenic-induced oxidative stress

Induction of ROS and depletion of antioxidant defenses by arsenic have shown to be one of the most important factors governing its toxic effects. A therapeutic strategy to increase the antioxidant capacity of cells may fortify the long-term effective treatment of arsenic poisoning. This may be accomplished by either reducing the possibility of the metal interacting with critical biomolecules or bolstering cells with the supplementation of antioxidant molecules. Listed below are antioxidants that have been explored for their role in reversing arsenic-induced oxidative stress and related disorders.



Scheme 1. Proposed structure-activity relationship of N-acetylcysteine [325].

#### Antioxidant supplementation (natural and synthetic)

##### N-acetylcysteine (Scheme 1)

NAC is a thiol, a mucolytic agent, and a precursor of L-cysteine and reduced glutathione. NAC is a sulfhydryl-containing antioxidant that has been used to mitigate various conditions of oxidative stress, is known to have metal-chelating properties [312,321], and has been used in several clinical conditions [322,323]. Martin et al. reported a better response of acute arsenic symptoms to intravenous NAC than to intramuscular 2,3-dimercapto-1-propanol [324]. We provided evidence of a novel therapeutic combination to achieve greater effectiveness during chelation of arsenic in rats by coadministering NAC during treatment with *meso*-2,3-dimercaptosuccinic acid (DMSA) [138,325]. Santra et al. reported a correlation between liver cell injury and oxidative stress in arsenic-exposed mice [115]. The perturbations in the mitochondrial redox state, apoptosis of hepatocytes, and all the related changes were reduced in intensity and modified by pretreatment with NAC. NAC could effectively replenish cellular stores of the tripeptide GSH and is an effective intervention against arsenic-induced oxidative stress.

##### $\alpha$ -Lipoic acid (LA) (Scheme 2)

$\alpha$ -Lipoic acid (LA) or 1,2-dithione-3-pentanoic acid, a naturally occurring antioxidant, functions as a cofactor in several multienzyme complexes [326]. Lipoic acid is active in both lipid and aqueous phases [325]. Its reduced form, dihydrolipoic acid (DHLA), has two free sulfhydryl groups, and the two forms LA/DHLA, possess a great antioxidant potential [327]. Studies have demonstrated superior antioxidant activity of DHLA compared to LA [325,328,329]. Lipoic acid has an advantage over NAC in addressing GSH loss, because LA is effective in a micromolar range, whereas millimolar NAC is required for getting the similar effects [330]. The antioxidant effects of LA are based on its interaction with peroxyl radicals, which are essential for the initiation of lipid peroxidation, and ascorbyl radicals of vitamin C. DHLA can recycle ascorbyl radicals and reduce dehydroascorbate generated in the course of ascorbate oxidation by radicals. DHLA may act as a strong chain-breaking antioxidant and may enhance the antioxidant potency of other antioxidants, such as vitamin C, in both aqueous and hydrophobic membrane phase. LA can also be neuroprotective in vivo because of suppression of glial reactivity [331]. Lipoic acid has the capability to interfere with the absorption of

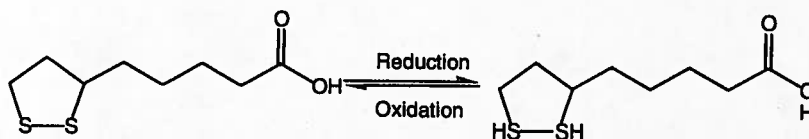
arsenic [327,332,333]. The protective effects of LA against arsenic-induced oxidative stress could be attributed to (a) the ability of LA to be reduced to dihydrolipoic acid by NADH; DHLA, being a strong antioxidant, scavenges excess oxidants and recycles other antioxidants such as vitamin E, vitamin C, and glutathione; (b) the ability of DHLA to chelate arsenic and prevent free radical generation, thus diminishing oxidant attacks on biomacromolecules; (c) LA being the key cofactor of pyruvate dehydrogenase and  $\alpha$ -ketoglutaric dehydrogenase, the enzymes sensitive to oxidative stress; (d) the ability of LA supplementation to stimulate the activities of enzymes, thereby promoting and ameliorating oxidative phosphorylation and mitochondrial respiration; and (e) the ability of LA to promote antioxidant defense by inducing phase II enzymes, such as glutathione synthetase, to elevate antioxidant GSH [325,330,334].

##### Vitamin E ( $\alpha$ -tocopherol) and vitamin C

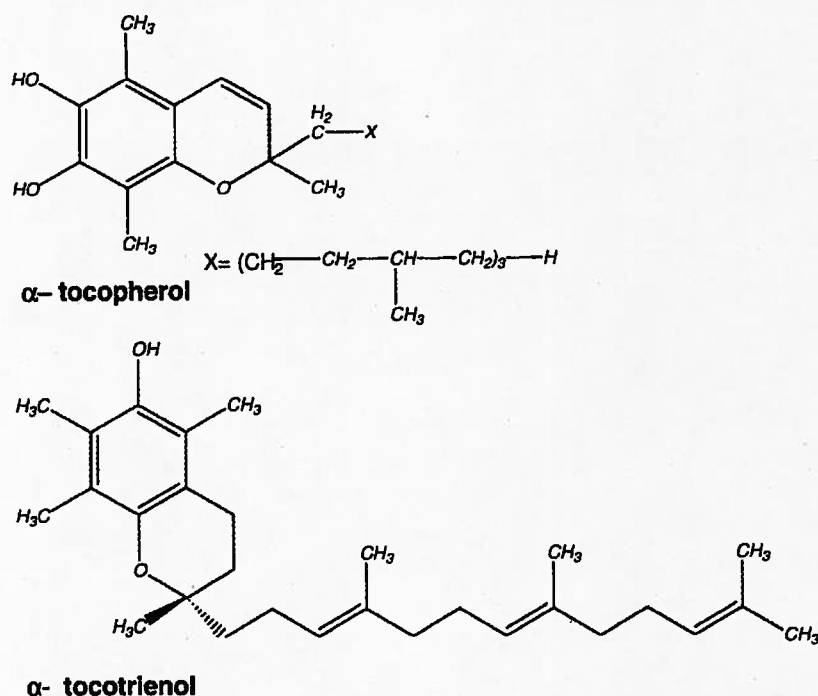
Vitamin E (Scheme 3) is the generic term used to describe at least eight naturally occurring compounds that possess the biological activity of  $\alpha$ -tocopherol [325,335]. Protective effects of vitamin E emerge directly from its antioxidant property and its influence on the drug-metabolizing enzyme system [336–338]. Vitamin E has shown an ability to protect against arsenic intoxication. It is believed that vitamin E, as a scavenger of free radicals, might react with methyl radicals that might be formed in the breakdown to provide protection. Vitamin E treatment reversed altered variables of the heme synthesis pathway, oxidative stress in liver and kidneys, and concentration of essential metals in the blood and soft tissues of Swiss albino male mice [339].

Vitamin C, a low-molecular-mass antioxidant, interacts directly with oxidizing radicals and protects the cells from reactive oxygen species in vivo and in vitro [340–342] (Scheme 4). Vitamin C scavenges aqueous ROS by rapid electron transfer that inhibits lipid peroxidation [343]. It acts mainly as an antioxidant molecule and its beneficial effects could be attributed to its ability to complex with arsenic. Administration of vitamin C or vitamin E in combination with a thiol chelator produced more pronounced recovery than the chelator alone in subchronically arsenic-exposed rats [344,345].

A few clinical studies conclude that intake of B vitamins and antioxidants at doses greater than the current recommended daily amounts for Bangladesh might lower the risk of arsenic-related skin lesions [346,347].



Scheme 2. Oxidized (LA) and reduced forms (DHLA) of lipoic acid [325].

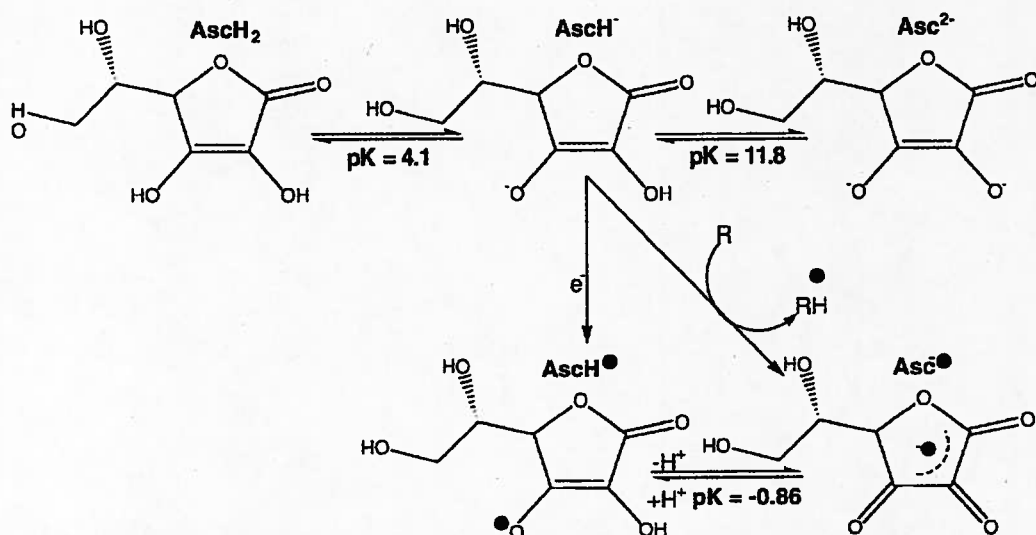
Scheme 3.  $\alpha$ -Tocopherol and  $\alpha$ -tocotrienol.**Taurine (Scheme 5)**

Taurine, or 2-aminoethanesulfonic acid, a sulfur-containing  $\beta$ -amino acid, has a role in maintaining calcium homeostasis, osmoregulation, removal of hypochlorous acid, and stabilizing membranes. Taurine acts directly by scavenging ROS and indirectly by preventing changes in membrane permeability due to oxidant injury. Roy et al. reported that it has the ability to ameliorate arsenic oxidative insult and renal damage, probably through antioxidant activity and functioning via MAPKs/NF- $\kappa$ B and mitochondria-dependent pathways [348]. Oral taurine administration is known to counteract arsenic-induced oxidative stress, attenuate testicular damage, and ameliorate apoptosis in testicular tissue by controlling the reciprocal regulation of Bcl-2/Bad, phospho-ERK1/2, phospho-p38, phospho-Akt, and NF- $\kappa$ B [349]. The combined administration of taurine with a thiol chelator reversed oxidative stress in

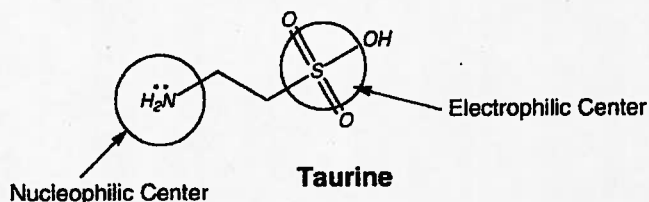
chronically arsenic-exposed rats [147]. An antioxidant mechanism, rather than a chelating activity, of taurine seems to underlie these beneficial effects.

**Quercetin (Scheme 6)**

The flavonol quercetin (3',3,4',5,7-pentahydroxyflavone), one of the most abundant dietary flavonoids, is found in fruits and vegetables, olive oil, red wine, and tea [325,350–352]. Quercetin scavenges free radicals and reduces the oxidizability and cytotoxic effects of low-density lipoproteins [353–356]. Its antiradical property scavenges hydroxyl and superoxide radicals, whereas the phenolic groups act as possible chelating sites [321]. Quercetin administration was also found to be associated with reduced conditions of oxidative stress induced by arsenic exposure when administered either alone or



Scheme 4. pH dependent reduction of ascorbic acid suggesting possible binding sites for chelation and antioxidant effects [325].



Scheme 5. Structure of taurine showing nucleophilic and electrophilic centers.

in combination with a thiol chelator [332,356]. Quercetin in a nanocapsulated drug delivery system provided better therapeutic efficacy than its bulk form at preventing arsenic-induced hepatic and cerebral oxidative damage [356,357].

#### Essential metals

Zinc is one of the essential trace metals and has been studied for its protective value against arsenic. Cellular zinc is stored in the cytosolic cysteine-rich protein metallothionein (MT), and homeostasis is maintained to keep free intracellular Zn(II) in the pico- to nanomolar range. Arsenic is capable of inducing MT, suggesting that this cysteine-rich low-molecular-weight protein might play a role in arsenic detoxification. A few studies were planned based on this theory, but provided data contradictory to the above. Kreppel et al. reported that zinc-induced increase in MT does not seem to be responsible for the protective role of preadministered zinc against arsenic-induced lethality [358]. A few other reports suggest that zinc pretreatment affords an increase in arsenic elimination. Iron or zinc, either individually or in combination with a thiol chelator, during and after arsenic exposure provided more pronounced elimination of arsenic in male mice [358,359]. Further, arsenic inhibits several zinc-dependent enzymes by replacing Zn(II) in the ZF motif (Section 9). Zinc supplementation thus has been proposed to prevent such damage that may chronically lead to manifestations such as cancer. However, arsenic-induced ZF inhibition may not be reversible as in the case of MMA<sup>III</sup>, whereas it may be reversible for As<sub>i</sub> binding [139,307]. It is clear from the above that not much work has been done on the role of zinc against arsenic. Studies with variable doses of zinc administered during chelation of arsenic, particularly against chronic arsenic poisoning, are recommended [360].

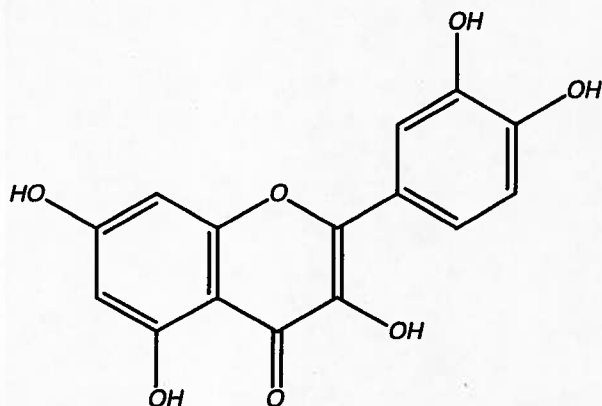
Selenium is known to promote biliary excretion of exogenous selenium, and selenite augments the excretion of arsenic into bile

[192,358]. These studies suggested that arsenic augmented the hepatobiliary transport of selenium and facilitated accumulation of selenium in red blood cells. Selenium in turn facilitated the biliary excretion of arsenic. Glattre et al. studied the distribution and interaction of arsenic and selenium in rat thyroid and suggested that both arsenic and selenium accumulate in thyroid tissue [361]. Competition between selenium and arsenic for binding with functional proteins, bioligands, and active tissue sites and the formation of a reversible compound, metal-selenide, are the proposed mechanisms that may lead to the reduction of available "free" arsenic ions in the body.

#### Natural/herbal antioxidants

A number of vegetables and plant parts or their extracts are gaining importance in reducing the toxic effects of arsenic. Coadministration of some plant extracts, such as curcumin, *Hippophae rhamnoides*, *Aloe vera barbadensis*, *Centella asiatica*, and *Allium sativum*, either with arsenic exposure or during chelation treatment, has shown beneficial effects on arsenic-induced hematological, renal, and hepatic variables in experimental animals [166,362–366]. Curcumin is a phytochemical from turmeric that was evaluated during a field trial conducted in West Bengal [367]. Seabuckthorn (*H. rhamnoides*, Elaeagnaceae) is a rich source of a large number of bioactive substances such as vitamins A, C, and E; carotenoids; and organic acids and its beneficial effects on arsenic toxicity have been attributed to the high content of antioxidant substances present in this plant [363,364]. *A. vera* (*A. barbadensis*) has limited protective value against arsenic-induced oxidative stress, but *C. asiatica* (Umbelliferae) syn *Hydrocotyl asiatica* has been shown to be beneficial in improving alterations in arsenic-induced oxidative stress [365]. It is also beneficial in a limited way in depleting tissue arsenic concentrations in arsenic-exposed animals [366]. *Moringa oleifera* (particularly the seed) is another plant product that has recently been reported to exhibit significant protection of altered biochemical variables in addition to being able to reduce liver and blood arsenic burden in exposed animals. The mechanism of such protection has been attributed to the interactions between cysteine- and methionine-rich proteins that are present in high amounts in *M. oleifera* seed powder and responsible for the removal of arsenic from in vivo sites [368–370].

Garlic contains a number of organosulfur compounds, which are widely believed to be active agents [371]. Coadministration of garlic extract is able to reduce the clastogenic effects of sodium arsenite [372,373]. These studies suggested the critical roles of p53 and heat shock proteins. Concomitant administration of garlic extract reduced tissue arsenic burden and increased urinary arsenic excretion. The protection was attributed to the thiosulfur components present in the garlic extract, which may act as Lewis acids and interact with a Lewis base (arsenic) to form stable components [166]. Amagase et al. and Chowdhury et al. suggested that diverse components of aqueous garlic extracts such as allicin also participate in arsenic chelation [374,375]. Sulfur-bearing components of aqueous garlic extract are lipophilic and thus easily permeate phospholipid membranes [376].



Scheme 6. Chemical structure of quercetin.

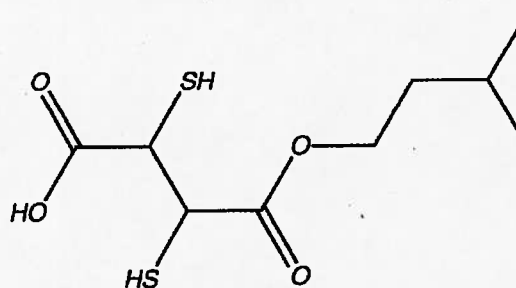
## Chelation therapy

Chelating agents are organic compounds capable of linking together metal ions to form complex ring-like structures called chelates. Sodium 2,3-dimercaptopropane 1-sulfonate (DMPS), DMSA (Scheme 7), and one of its analogues, monoisoamyl-DMSA (MiADMSA), are effective chelators, and the dithiol group in their moieties acts as an oxygen radical scavenger, which helps in inhibiting lipid peroxidation [377–380]. DMSA and DMPS are effective in reversing arsenic-induced toxicity based on enhanced urinary arsenic excretion and restoration of inhibited blood ALAD activity and hepatic glutathione [191,381].

We reported a significant depletion of arsenic and significant recovery of the altered biochemical variables indicative of oxidative stress and ALAD in chronically arsenic-exposed rats. Inhibition of ALAD activity is known to cause accumulation of ALA, which may autoxidize to generate reactive oxygen species [382]. Altered SOD activity, GSH level, and ALAD activity all responded favorably to the chelation therapy. Decreased GSH:GSSG ratio, which is indicative of a disturbance in the delicate antioxidant/pro-oxidant balance, also responded favorably to MiADMSA. Chelation therapy with MiADMSA was able to counteract arsenic-induced oxidative stress but exhibited some hepatotoxic effect as indicated by increased serum AST activity [383,384]. In vivo evidence of arsenic-induced oxidative stress in a number of major organs of arsenic-exposed rats and the effects being mitigated by pharmacological intervention encompassing combined treatment with *N*-acetylcysteine and DMSA has been reported [142].

A large number of esters of DMSA have been synthesized to achieve better chelation compared to DMSA. These esters are mainly the mono and dimethyl esters of DMSA that have been studied experimentally with the aim of enhancing tissue uptake of chelating agents [385–390]. DMSA analogues are capable of crossing membranes and are more effective in reducing arsenic in acute and subchronic exposure [389,390].

Among these new chelators, the monoisoamyl ester of DMSA (MiADMSA; a C<sub>5</sub> branched-chain alkyl monoester of DMSA; Scheme 8) has been found to be the most effective at reducing arsenic burden. We compared various chelating agents (three amino and four thiol chelators) on their roles in metal redistribution, hepatotoxicity, and oxidative stress from chelator-induced metallothionein in rats [384]. Among the seven chelators, MiADMSA and DMSA produced the least oxidative stress and toxicity compared to all the other chelators. Except for moderate developmental toxicity no major toxic effects so far have been reported with this chelator [391,392]. Taubeneck et al. showed that the developmental toxicity of DMSA is mediated mainly through disturbed copper metabolism and this may also hold for MiADMSA [393]. Toxicity profiles of MiADMSA in male and female rats were reported and no major alterations in the heme biosynthesis pathway, except for a slight rise in the zinc protoporphyrin, were seen [394,395]. MiADMSA produced moderate maternal oxidative stress at higher doses. MiADMSA administration caused some changes in the essential metal concentration in the soft tissues, especially loss of copper in lactating mothers and pups [396,397]. Dose- and route-dependent efficacy of MiADMSA against chronic arsenic poisoning has



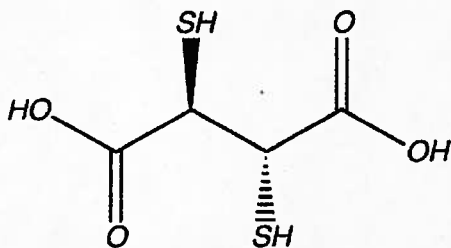
Scheme 8. Monoisoamylthiomersucinic acid.

also been suggested. This chelator is highly effective through the oral route at reversing arsenic-induced changes in the variables indicative of oxidative stress in major organs and arsenic mobilization [100,398]. The data suggest that MiADMSA may be a future drug of choice owing to its lipophilic character and the absence of any metal redistribution [399]. Moderate toxicity after repeated administration of MiADMSA may be reversible after withdrawal of the chelating agent or use of an antioxidant during chelation treatment.

## Conclusion and future strategies

The above discussion points to the fact that reactive oxygen species play the most crucial role in exerting the toxic effects of arsenic on various tissues. Introduction of arsenic into a cell causes an imbalance between pro-oxidants and endogenous antioxidants that triggers various pathways to prime the cell toward apoptosis or immortality. Although the mechanism of arsenic-induced toxicity still remains poorly understood, the mitochondria are presumed to be one of the major targets for the generation of reactive species, which trigger a cascade of events leading to cell death. The action of arsenic-induced apoptosis is complex and H<sub>2</sub>O<sub>2</sub> is believed to be involved in the process. It plays a role as a mediator to induce apoptosis through the release of cytochrome *c* to the cytosol, activation of CPP32 protease, and PARP degradation. There has also been report of MAPK pathways contributing to cell growth regulation and cell death during arsenic exposure. Arsenic triggers various signaling pathways that control important functions such as proliferation, differentiation, and apoptosis. Arsenic detection in biological samples is the most common indication of exposure; analysis of various macromolecules and ROS levels can also provide indications of arsenic-inflicted toxicity. Future studies involving microarray-based gene expression analysis might be able to provide better insight into delineating the exact mechanisms of action and identifying the target genes.

It is thus evident that there is enough experimental evidence available in the literature clearly suggesting that oxidative stress plays an important role in the molecular mechanism of arsenic-induced toxicity and related diseases. Exposure to arsenic generates O<sub>2</sub><sup>•−</sup>, which is converted to various other reactive species such as <sup>•</sup>OH radical. The reaction and interaction of these reactive species with target molecules lead to oxidative stress, DNA damage, and activation of signaling cascades associated with promotion and progression of tumor. 8-OHdG has been proposed to be an excellent marker of arsenic-induced oxidative stress on DNA. Increased levels of 8-OHdG have been reported in arsenic-exposed animal models. Arsenic induces DNA adducts through calcium-mediated production of peroxynitrite, hypochlorous acid, and hydroxyl radicals. Various reactive species pathways discussed in this review could be the mechanisms of action of arsenic-induced human carcinogenicity. In summary, arsenic-induced oxidative stress leading to apoptosis can be attributed mainly to (i) depletion of intracellular glutathione, (ii) Bax/Bak-dependent release, (iii) alterations in intracellular PKC activation and calcium, (iv) activation of c-jun-N



Scheme 7. Meso-2,3-dimercaptosuccinic acid.

terminal kinase, (v) DNA strand breaks in human fibroblasts, and (v) increased levels of 8-OHdG and activation of AP-1 and NF- $\kappa$ B.

Various antioxidants are known to protect cells from oxidative injury; however, the effectiveness of thiol-chelating agents in reversing these changes is still an area of debate and future investigation. Because the toxic manifestation of arsenic is mainly mediated through the generation of ROS and RNS and their direct binding with SH groups, combination therapy with an appropriate antioxidant (such as NAC) and a lipophilic thiol chelator could be a major future strategy to treat cases of chronic arsenic poisoning.

Chelation therapy remains the mainstay of arsenicosis, despite the lack of arsenic-specific chelating agents. Development of lipophilic, nontoxic chelators has opened new doors in the area of treatment of arsenic poisoning. Monotherapy with chelators may not be beneficial in providing better clinical recoveries. Thus combination therapy utilizing antioxidants such as NAC, taurine, or herbal extracts with a thiol-chelating agent will be beneficial. Use of two structurally different chelating agents, too, can be a useful strategy. We recommend that analogues having a long carbon chain (e.g., MIADMSA) are better chelators than short-carbon-chain chelators. Analogues of DMSA simultaneously eliminate arsenic from the cell and provide assistance in bringing GSH homeostasis toward normalcy, and combinational therapy with a lipophilic and hydrophobic chelator shows more promise than the use of a single chelator.

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# Arsenic: toxicity, oxidative stress and human disease

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**ABSTRACT:** Arsenic (As) is a toxic metalloid element that is present in air, water and soil. Inorganic arsenic tends to be more toxic than organic arsenic. Examples of methylated organic arsenicals include monomethylarsonic acid [MMA(V)] and dimethylarsinic acid [DMA(V)]. Reactive oxygen species (ROS)-mediated oxidative damage is a common denominator in arsenic pathogenesis. In addition, arsenic induces morphological changes in the integrity of mitochondria. Cascade mechanisms of free radical formation derived from the superoxide radical, combined with glutathione-depleting agents, increase the sensitivity of cells to arsenic toxicity. When both humans and animals are exposed to arsenic, they experience an increased formation of ROS/RNS, including peroxy radicals (ROO<sup>•</sup>), the superoxide radical, singlet oxygen, hydroxyl radical (OH<sup>•</sup>) via the Fenton reaction, hydrogen peroxide, the dimethylarsenic radical, the dimethylarsenic peroxy radical and/or oxidant-induced DNA damage. Arsenic induces the formation of oxidized lipids which in turn generate several bioactive molecules (ROS, peroxides and isoprostanes), of which aldehydes [malondialdehyde (MDA) and 4-hydroxy-nonenal (HNE)] are the major end products. This review discusses aspects of chronic and acute exposures of arsenic in the etiology of cancer, cardiovascular disease (hypertension and atherosclerosis), neurological disorders, gastrointestinal disturbances, liver disease and renal disease, reproductive health effects, dermal changes and other health disorders. The role of antioxidant defence systems against arsenic toxicity is also discussed. Consideration is given to the role of vitamin C (ascorbic acid), vitamin E ( $\alpha$ -tocopherol), curcumin, glutathione and antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in their protective roles against arsenic-induced oxidative stress. Copyright © 2011 John Wiley & Sons, Ltd.

**Keywords:** arsenic; oxidative stress; free radicals; ROS; RNS; human disease; antioxidants

## INTRODUCTION

Arsenic is the 33rd element of the Periodic Table of the chemical elements, and while it is classified formally as a metalloid, meaning that it displays some properties of both a metal and a nonmetal, it is frequently also referred to as a metal and in the context of toxicology as a heavy metal (Mandal and Suzuki, 2002). Arsenic exists in nature in three allotropic forms,  $\alpha$  (yellow),  $\beta$  (black),  $\gamma$  (grey), of the metallic state and in a number of ionic forms. The most common oxidation numbers of arsenic are +5, +3 and -3, in which the element is able to form both inorganic and organic compounds both in the environment and within the human body (Orloff *et al.*, 2009). In combination with other elements such as oxygen, sulfur and chlorine, the element is referred to as *inorganic arsenic* and as combined with hydrogen and carbon as *organic arsenic*. Since most arsenic compounds lack colour or smell, the presence of arsenic is not immediately obvious in food, water or air, thus presenting a serious human health hazard given the toxic nature of the element. Indeed, the very name arsenic is synonymous with poison, in consequence of its long and nefarious history (Mandal and Suzuki, 2002). Arsenic is ubiquitous in nature and its abundance ranks twentieth in the Earth's crust, fourteenth in seawater and twelfth in the human body.

## SOURCES AND ROUTES OF EXPOSURE TO ARSENIC

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) is the most prevalent inorganic arsenical found in air, while a variety of inorganic arsenates (AsO<sub>4</sub><sup>3-</sup>) or

arsenites (AsO<sub>2</sub><sup>-</sup>) occur in water, soil or food (Magalhaes, 2002; Chou *et al.*, 2007). In consequence of its widespread use in the microelectronics industry, gallium arsenide (GaAs) is an inorganic arsenic compound which may also impact adversely on human health.

The largest source of arsenic and other metals is usually food, of which the main dietary forms are seafood, rice, mushrooms and poultry (Jones, 2007; Petroczi and Naughton, 2009; Nepusz *et al.*, 2009; Smedley and Kinniburgh, 2002). While there is more arsenic *per se* in seafood, this is mostly in an organic form called arsenobetaine which is much less harmful than others. Mostly, arsenic poisoning occurs through industrial exposure, from contaminated wine or moonshine, or by malicious administration.

Very recently, it has been reported that traditional Chinese herbal products, deliberately fortified with arsenic for therapeutic

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purposes, may represent a serious health hazard (Martena *et al.*, 2010). Mass spectrometry has revealed that, of 292 tested samples sold in Dutch market, 20% significantly exceeded safety levels not only of arsenic but also of lead and mercury. It has been concluded that traditional herbal preparations of Chinese and Tibetan medicine require strict control by local authorities.

Colour pigments that are used in the cosmetic industry in the production of eye-shadows frequently contain toxic elements, including arsenic (Sainio *et al.*, 2000). The skin of the eyelids is very delicate and the application of eye-shadows may produce eczemas. In addition, arsenic particles can be water soluble and therefore may undergo percutaneous absorption through the wet skin. When it enters the circulatory system via percutaneous absorption, at high concentrations, arsenic may represent a potential risk of carcinogenesis. Based on the available toxicology data, it has been recommended that cosmetic products should contain less than 5 ppm of metal impurities.

As contained in water, soil or food, ingested arsenic may quickly enter the human body. When air containing arsenic dusts is breathed in, the majority of the dust particles settle onto the lining of the lungs (Chen *et al.*, 2006). Very little internal exposure to arsenic occurs via the material passing through the skin into the body, and so there is little risk of arsenic poisoning posed by this route.

The majority of arsenic enters the body in the trivalent inorganic form As(III) via a simple diffusion mechanism (Cohen *et al.*, 2006). Only a small amount of pentavalent inorganic arsenic can cross cell membranes via an energy-dependent transport system, after which it is immediately reduced to trivalent arsenic.

Both organic and inorganic forms of arsenic leave the body in urine and thus most inorganic arsenic will be expelled after several days, although some will remain for a number of months or even longer (Aposhian *et al.*, 2000a, b). The majority of organic arsenic is expelled more rapidly and usually within several days.

Groundwater contamination by arsenic and other metals has impacted severely on the health of the populations of various regions in the world. Some of the most profound examples of contamination by arsenic occur in Bangladesh and West Bengal, in India, where it has been discovered that almost 43 million people have been drinking water that is laden with arsenic (Chowdhury *et al.*, 2000). To place this in perspective, the WHO recommended limit for arsenic in water is  $10 \mu\text{g l}^{-1}$  (WHO factsheet no. 210, May 2001), while concentrations in the range  $50\text{--}3200 \mu\text{g l}^{-1}$  have been measured (Bhattacharya *et al.*, 2003). Table 1 summarizes the levels of arsenic in human tissues and urine collected from residents from the arsenic-contaminated areas.

**Table 1.** Levels of As in human urine and tissues of the residents from the arsenic-polluted areas<sup>a</sup>

Country/town or region	Concentration of arsenic/source of sample	References
UK/Glasgow	0.650 $\mu\text{g g}^{-1}$ (hair) 0.048 $\mu\text{g g}^{-1}$ (liver) 0.015 $\mu\text{g g}^{-1}$ (spleen) 0.007 $\mu\text{g g}^{-1}$ (lung)	Rale (1996)
Germany/Nürnberg, Saxony	5.5 $\text{ng g}^{-1}$ (lung) 7.58 $\mu\text{g g}^{-1}$ (urine)	Kraus <i>et al.</i> (2000) Gebel <i>et al.</i> (1998)
Turkey/Ismir	4.23 $\mu\text{g l}^{-1}$ (breast milk)	Ulman <i>et al.</i> (1998)
Mexico/Hermosa		
Concentration of As in water:		
9 $\mu\text{g l}^{-1}$	10.26 $\mu\text{g}$ per day (urine, 24 h)	Wyatt <i>et al.</i> (1998)
15 $\mu\text{g l}^{-1}$	10.54 $\mu\text{g}$ per day (urine, 24 h)	
30 $\mu\text{g l}^{-1}$	25.18 $\mu\text{g}$ per day (urine, 24 h)	
USA/Fort Valley	11.6 $\mu\text{g l}^{-1}$ (urine, randomly collected) 11.0 $\mu\text{g l}^{-1}$ (urine, 24 h) 0.78 $\mu\text{g g}^{-1}$ (hair)	Hewitt <i>et al.</i> (1995)
Middleport	15.1 $\mu\text{g l}^{-1}$ (urine) (children <7 years) 15.7 $\mu\text{g l}^{-1}$ (urine) (children <13 years) 15.7 $\mu\text{g l}^{-1}$ (urine) (adults)	Tsuji <i>et al.</i> (2005)
Spain/Catalania	<0.05 $\mu\text{g g}^{-1}$ (lung) <0.05 $\mu\text{g g}^{-1}$ (bone) <0.05 $\mu\text{g g}^{-1}$ (kidney) <0.05 $\mu\text{g g}^{-1}$ (liver)	Garcia <i>et al.</i> (2001)
India/West Bengal	7.32 $\mu\text{g g}^{-1}$ (finger nails) 4.46 $\mu\text{g g}^{-1}$ (hair)	Mandal <i>et al.</i> (2003)

<sup>a</sup>Adapted from Chou *et al.* (2007).

## TOXICITY OF ARSENIC

Inorganic arsenic includes arsenite [As(III)] and arsenate [As(V)] and can be either methylated to form monomethylarsonic acid [MMA(V)] or dimethylated as in dimethylarsinic acid [DMA(V)]. The metabolism of inorganic arsenic involves a two-electron reduction of pentavalent arsenic to trivalent arsenic, mediated by glutathione, followed by oxidative methylation to form pentavalent organic arsenic (Fig. 1; Hughes, 2002).

Inorganic arsenic tends to be far more toxic than organic arsenic (Shi *et al.*, 2004; Valko *et al.*, 2005). Arsenic is toxic to the majority of organ systems, the most sensitive target organ being the kidney (Cohen *et al.*, 2006; see Table 1). The extent of arsenic poisoning depends on various factors such as dose, individual susceptibility to arsenic and the age of the affected individuals. While chronic arsenic exposure affects the vascular system and causes hypertension and cardiovascular disease, acute arsenic toxicity may cause cardiomyopathy and hypotension. The most common neurological effect of long-term arsenic toxicity is peripheral neuropathy and the gastrointestinal effects are manifested by toxic hepatitis accompanied by increased levels of liver enzymes.

Trivalent inorganic arsenic inhibits pyruvate dehydrogenase by binding to the sulfhydryl groups of dihydrolipoamide, resulting in a reduced conversion of pyruvate to acetyl coenzyme A (CoA), while both citric acid cycle activity and production of cellular ATP are decreased (Bergquist *et al.*, 2009). Trivalent arsenic inhibits numerous other cellular enzymes through sulfhydryl group binding. It also inhibits the uptake of glucose into cells, gluconeogenesis, fatty acid oxidation and further production of acetyl CoA. Significant to oxidative stress is that trivalent arsenic inhibits the production of glutathione, which protects cells against oxidative damage (Miller *et al.*, 2002).

In part, the toxicity of pentavalent inorganic arsenic is due to its conversion to trivalent arsenic, from which the toxic effects proceed as outlined above. At a more significant and specific level, pentavalent arsenic emulates inorganic phosphate and replaces

phosphate in glycolytic and cellular respiration pathways (Hughes, 2002). Uncoupling of oxidative phosphorylation occurs because the normal high-energy phosphate bonds are not formed; e.g. in the presence of pentavalent arsenic, adenosine diphosphate (ADP) forms ADP-arsenate instead of ATP with the absence of the high-energy ATP phosphate bonds.

The methylation of inorganic arsenic has been considered to be a detoxification mechanism (Aposhian, 1997). However, recent experimental results have documented the presence of trivalent intermediates, monomethylarsonous acid [MMA(III)] and dimethylarsinous acid [DMA(III)] in the urine of humans exposed to drinking water containing high levels of inorganic arsenic (Cohen *et al.*, 2006). These trivalent intermediates are structurally different from the pentavalent compounds and are more reactive and more carcinogenic.

## BIOMARKERS OF ARSENIC

### Quantification of the Exposure to Arsenic

Measurements of the level of arsenic in blood, urine, hair and nails have all been used as biological indicators of exposure to arsenic (Vahter, 1983). Since arsenic is metabolized from blood within a period of several hours (Tam *et al.*, 1979), the measurement of blood arsenic levels is not a good indicator of long-term exposure of individuals to arsenic.

On the basis that no correlation was found between the level of arsenic in the blood and the level of arsenic in the drinking water of residents in several communities in the USA, where water levels ranged from about 6 to 125  $\mu\text{g l}^{-1}$  (Valentine *et al.*, 1979), it was concluded that measurement of blood arsenic is not a reliable marker for arsenic exposure. We may note that typical background values of blood concentration for Arsenic in nonexposed individuals are  $<1 \mu\text{g l}^{-1}$  (Heydorn, 1970), while blood levels in acutely toxic and fatal cases may be 1000  $\mu\text{g l}^{-1}$  or even greater (Driesback, 1980).

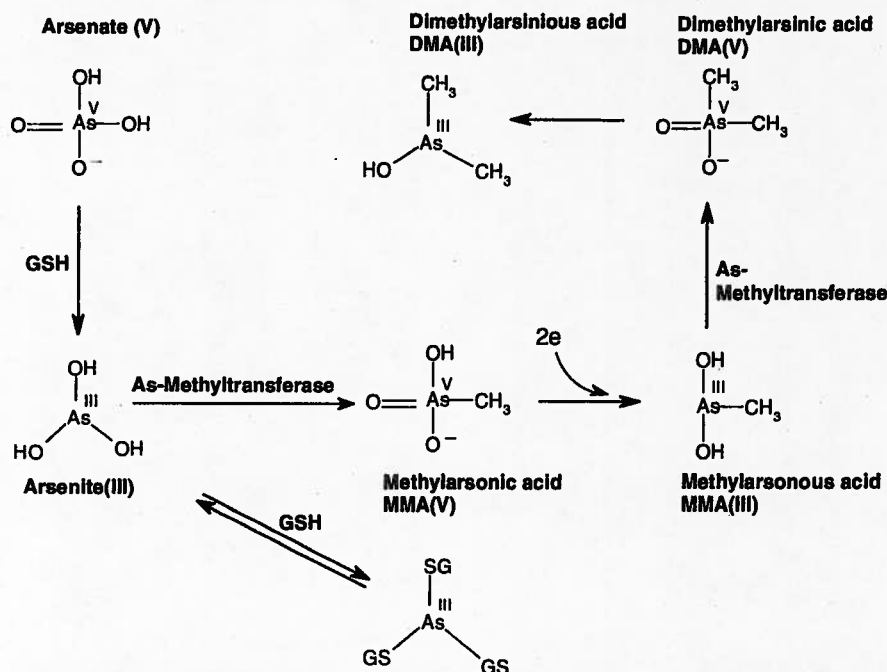


Figure 1. The metabolism of inorganic arsenic.

Since the majority of arsenic is absorbed from the lungs or the gastrointestinal tract and excreted in the urine, normally within 1–2 days, measurement of urinary arsenic levels is generally considered as the most reliable marker of acute arsenic exposure (Polissar *et al.*, 1990). Indeed, such measurements are found to correlate well with exposures of populations living near industrial point sources of arsenic (see for example Milham and Strong, 1974).

### Biomarkers of the Effect Caused by Arsenic

Arsenic is known to influence the activity of a number of enzymes, in particular the group of enzymes responsible for heme synthesis and degradation (Woods and Fowler, 1978) and activation of heme oxygenase (Sardana *et al.*, 1981). Menzel and coworkers (Menzel *et al.*, 1998) have also examined the *in vitro* induction of human lymphocyte heme oxygenase 1 (HO1) as a biomarker of arsenite exposure. Arsenite was observed to induce *de novo* synthesis of HO1 in human lymphoblastoid cells, but it has not been determined whether the same response is induced *in vivo*.

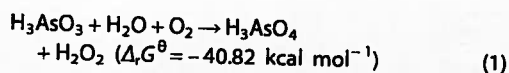
Animal tests have shown that arsenic poisoning increased urinary levels of uroporphyrin, coproporphyrin and bilirubin (Albores *et al.*, 1989). These tests have also been shown to be applicable to human subjects (García-Vargas and Hernández-Zavala, 1996). Hence, altered urinary levels of these heme-related compounds could serve as a sensitive biomarker of the effect of arsenic.

### ARSENIC AND OXIDATIVE AND NITROSATIVE STRESS

Many mechanistic studies of arsenic toxicity have suggested that reactive oxygen species and reactive nitrogen species are generated during inorganic arsenic metabolism in living cells (Shi *et al.*, 2004).

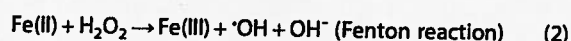
Arsenic induces morphologic changes in mitochondrial integrity and a rapid decline of mitochondrial membrane potential. Mitochondrial alterations are considered to be primary sites where an uncontrolled random formation of superoxide anion radical occurs. Cascade mechanisms of free radical formation derived from the superoxide radical combined with a decrease in cellular oxidant defence by treatment with glutathione-depleting agents results in an increased sensitivity of cells to arsenic toxicity (Cohen *et al.*, 2006; Valko *et al.*, 2005). Experimental results based on both *in vivo* and *in vitro* studies of arsenic-exposed humans and animals suggest the possible involvement of increased formation of peroxyl radicals (ROO<sup>•</sup>), superoxide anion radical (O<sub>2</sub><sup>•-</sup>), singlet oxygen (<sup>1</sup>O<sub>2</sub>), hydroxyl radical (<sup>•</sup>OH), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), dimethylarsenic radical [(CH<sub>3</sub>)<sub>2</sub>As<sup>•</sup>], blood nonprotein sulfhydryls and/or oxidant-induced DNA damage (Flora *et al.*, 2007). The exact mechanism responsible for the generation of all these reactive species has yet to be fully elucidated, but some studies have proposed the formation of intermediary arsine species.

An interesting route to H<sub>2</sub>O<sub>2</sub> production was proposed to involve the oxidation of As(III) to As(V) which, under physiological conditions, results in the formation of H<sub>2</sub>O<sub>2</sub> (Valko *et al.*, 2005 and references therein).



The above reaction is spontaneous and exergonic with an estimated standard reaction free energy change for H<sub>2</sub>O<sub>2</sub> formation of  $-40.82 \text{ kcal mol}^{-1}$  ( $-170.87 \text{ kJ mol}^{-1}$ ).

Hydrogen peroxide is not a free radical species; however, when an organism is overloaded by iron (as in the conditions of haemochromatosis, haemolytic anemias and haemodialysis), the high cellular concentrations of 'free available iron' may have deleterious effects, as is demonstrated by the participation of Fe (II) in the decomposition of hydrogen peroxide (Fenton reaction), generating highly reactive hydroxyl radicals:



The hydroxyl radical is a highly reactive ROS with a half-life shorter than 1 ns in an aqueous environment (Valko *et al.*, 2007). Thus when it is produced *in vivo* it reacts in regions close to its site of formation. The formation of <sup>•</sup>OH in the vicinity of DNA might lead to this radical reacting with DNA bases or with the deoxyribose backbone of DNA to produce modified (damaged) bases or strand breaks. The majority of the hydroxyl radicals generated *in vivo* arise from the metal catalysed decomposition of hydrogen peroxide, according to the Fenton reaction (Naughton *et al.*, 1993).

In addition to reactive oxygen species, arsenic exposure can initiate the generation of reactive nitrogen species (RNS). Several contradictory results describing arsenic-induced production of NO<sup>•</sup> have been reported, one of which concluded that there was no arsenic-induced increase in NO<sup>•</sup> generation in hepatocytes and human liver cells, which inhibited inducible NO synthase gene expression in cytokine-stimulated human liver cells and hepatocytes (Hughes, 2002; Flora *et al.*, 2008; Germolec *et al.*, 1996). However, in another study, arsenite was said to inhibit inducible NO synthase gene expression in rat pulmonary artery smooth muscle cells (Kodavanti *et al.*, 1998). A third study with low levels of arsenite (<5 μM) similarly recorded no change in intracellular concentration of Ca(II), nor any NO<sup>•</sup> generation, according to results from EPR spectroscopy (Barchowsky *et al.*, 1999).

### GENOTOXICITY OF ARSENIC

There have been a large number of *in vitro* and *in vivo* studies made, devoted to determining the genotoxicity of inorganic arsenicals (Yamanaka *et al.*, 2004; Cohen *et al.*, 2006). *In vitro* studies on human fibroblasts, leukocytes, lymphocytes and hamster embryo cells have shown that arsenic induces chromosomal aberrations and sister chromatid exchange (Helleday *et al.*, 2000). Similar studies using human, mouse and hamster cells explored a potential enhancement of DNA damage, DNA repair enhancement or the inhibition of DNA synthesis.

Studies of humans have detected a higher than average incidence of chromosomal aberrations in peripheral lymphocytes, after both inhalation exposure (Nordenson *et al.*, 1978) and oral exposure (Nordenson *et al.*, 1979). These studies must be interpreted with caution, since in most cases, there were only a small number of subjects and influences from exposure to other chemicals could also affect the results.

Investigations of genotoxic effects of ingested arsenic in Taiwanese residents have yielded mixed results, possibly due to the different types of cells being examined and the different exposure levels experienced by the populations studied. Arsenic-related skin cancer has shown an accompanying much

higher rate in p53 mutations in comparison with those found in UV-induced skin cancer (Hsu *et al.*, 1999).

Occupational exposure of arsenic among workers in a glass plant in India whose levels of blood arsenic were five times higher than in the control group was reported to lead to increased DNA damage in leukocytes (Vuyyuri *et al.*, 2006).

An increased occurrence of chromosomal abnormalities was detected in rats given oral doses of sodium arsenate (4 mg As kg<sup>-1</sup> per day) for 2–3 weeks (Datta *et al.*, 1986). However, no increase in chromosomal aberrations was detected in bone marrow cells or spermatogonia from mice given sodium arsenite for a period of 2 months (Poma *et al.*, 1987). These studies suggest that ingested arsenic may cause chromosomal effects, but the data really are too limited to extract any firm conclusions.

The genotoxicity of organic arsenic has also been thoroughly investigated (see for example Kuroda *et al.*, 2004). DMA causes several genotoxic effects, including single strand DNA breaks, the formation of apurinic and apyrimidinic sites, an enhancement in oxidative stress as documented by oxidation of DNA bases, formation of DNA–protein crosslinks and chromosomal aberrations (Kitchin, 2001). Clastogenic effects of arsenic have been attributed to the high affinity of arsenic to sulfhydryl groups of proteins. Several tests indicate that not only DMA but also roxarsone (3-nitro, 4-hydroxyphenylarsonic acid) may be able to cause mutations and DNA strand breaks. *In vitro* studies with MMA did not find significant increases in the occurrence of chromosome aberrations, mutations or unscheduled DNA synthesis. In addition, an increased number of DNA strand breaks was detected in lung and other tissues of mice and rats given oral doses of ~1500 mg kg<sup>-1</sup> DMA (Okada and Yamanaka, 1994); this effect appeared to be related to the formation of some active oxygen species. Since the breaks were largely repaired within 24 h, the relevance related to any health risk is uncertain.

A study of p53 mutations in arsenic-related skin cancers from patients in Taiwan exposed to arsenic from drinking water found a high rate of p53 mutations and different types of p53 mutations compared with those seen in UV-induced skin cancers; similar results have been found in mice (Salim *et al.*, 2003).

While some animal studies have shown an increased incidence of chromosomal abnormalities in rats given oral doses of sodium arsenate for several weeks (Datta *et al.*, 1986), other studies did not confirm any consistent increase in chromosomal aberrations detected in bone marrow cells or spermatogonia in mice given sodium arsenite (Poma *et al.*, 1987). Thus the available data are too limited to draw a solid conclusion.

The most extensively studied DNA lesion is the formation of 8-OH-G, one of the major products of DNA oxidation, which originates from the reaction of hydroxyl radical with guanine (Fig. 2; Valko *et al.*, 2006). 8-OH-G is a sensitive genotoxic marker of oxidatively damaged DNA. Associations with increased urinary 8-OH-G concentrations have been seen also for arsenic exposure.

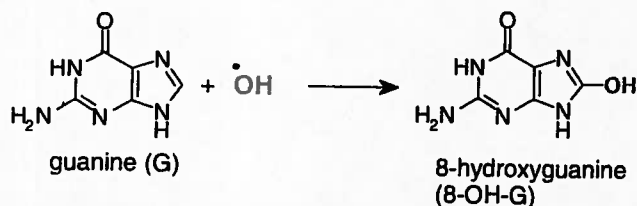


Figure 2. Reaction of guanine base (G) with hydroxyl radical.

As described above, arsenic is metabolized via methylation. A high concentration in urine, of the monomethylated As metabolite, methylarsonic acid (MMA), which is a susceptibility factor for As-induced toxicity, including carcinogenicity, has been correlated with similarly high urinary concentrations of 8-OH-G (Hu *et al.*, 2006). Interestingly, based on clinical trials, for a wide range of As exposure with urinary-As concentrations up to 1200 µg l<sup>-1</sup>, accepting the known pro-oxidative effects of As, the association of 8-OH-G with urinary-As was shown to be weaker than that for moderate exposure to cadmium. Thus, 8-OH-G may not be as sensitive a biomarker for As-induced oxidative stress as it is for Cd and for oxidative stress induced by other metals (Engström *et al.*, 2010).

## ARSENIC AND HUMAN DISEASE

Arsenic-induced genotoxicity may involve an alteration of the integrity of the cellular genetic material by oxidants or free radical species. Many recent studies have provided experimental evidence that arsenic-induced generation of free radicals and oxidative stress can cause cell damage and cell death through activation of oxidative sensitive signalling pathways (Fig. 3; De Vizcaya-Ruiz *et al.*, 2009). Arsenic exposure has been linked with various types of cancer (Miller *et al.*, 2002), cardiovascular disease (Navas-Acien *et al.*, 2005), diabetes (Díaz-Villaseñor *et al.*, 2007), neurological disorders (Vahidnia *et al.*, 2007) and dermal effects (Cohen *et al.*, 2006).

### Dermal Disease

Chronic exposure to arsenic leads to the development of lesions on the skin, including hyperkeratosis and hyperpigmentation, often used as diagnostic criteria for arsenicosis. (McCarty *et al.*, 2007). Dermal effects following the exposure to arsenic are hallmarks of the early stages of arsenic poisoning. Arsenic-induced cancers may appear later, sometimes taking several decades to develop symptoms (Lage *et al.*, 2006).

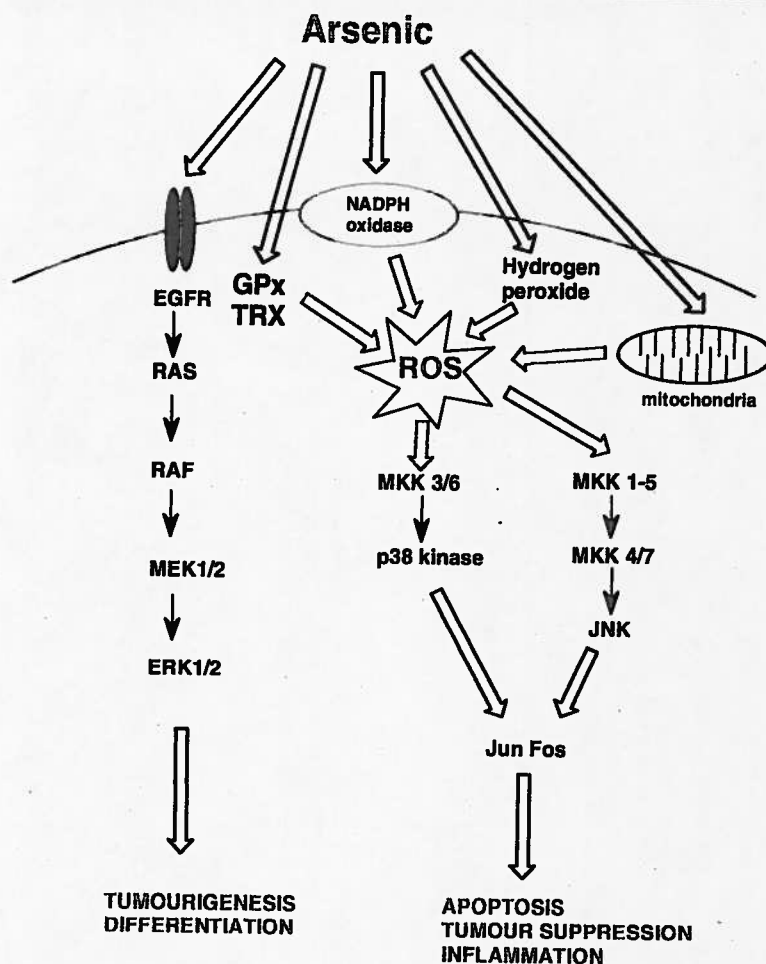
Workers exposed to inorganic arsenic in the air suffered from contact dermatitis and mild dermal irritation. Similar dermal effects (hyperkeratosis and hyperpigmentation) have been observed by the oral route of exposure among farmers in Taiwan who had been drinking arsenic-contaminated well water. The author of the trial stated that occurrence of dermal lesions was found to increase with dose (Tseng, 1977). Low, medium and high exposure levels corresponded to doses of 0.0008, 0.014, 0.038 and 0.065 mg As kg<sup>-1</sup> per day, respectively.

There is limited data accumulated for humans exposed to organic arsenic in air. Keratosis was observed in female workers in a chemical plant who were exposed to aersanilic acid (0.065 mg m<sup>-3</sup>; Chou *et al.*, 2007). Animal studies have shown that rats exposed to DMA (6 mg m<sup>-3</sup>) developed erythematous lesions on the feet and ears.

Oral exposure to organic arsenicals (MMA) with respect to dermal effects in humans has not been studied. Animal studies using rats and mice reported no histological skin alterations following chronic exposure to MMA.

### Cancer

Arsenic is a pernicious environmental carcinogen, and leads mainly to cancers of the skin, albeit that there is epidemiological evidence for lung, bladder, liver and kidney cancers being



**Figure 3.** Arsenic-mediated cell signalling (EGFR, epidermal growth factor receptor; Ras, GPx, glutathione peroxidase; Trx, thioredoxin; JNK, c-Jun N-terminal kinases, MKK, Mitogen-activated protein kinase kinases).

caused by exposure to arsenic (Rossman, 2003). It is thought that the mechanism by which these cancers originate may involve the promotion of oxidative stress by arsenic compounds, in which the antioxidant capacity of the living organism is overwhelmed by ROS (reactive oxygen species), resulting in molecular damage to proteins, lipids and most significantly DNA (Liu *et al.*, 2001).

Trivalent arsenic has been demonstrated to exhibit a greater toxicity than the corresponding pentavalent forms, in addition to a far more pronounced ability to release iron from the iron-storage protein ferritin (Salnikow and Zhitkovich, 2008). Free iron may catalyse the decomposition of hydrogen peroxide via the Fenton reaction, thereby forming the reactive hydroxyl radical which can cause DNA damage.

A large number of epidemiological trials have reported that inhalation exposure to inorganic arsenic increases the risk of lung cancer. An increased incidence of lung cancer has been reported among workers exposed primarily to arsenic trioxide dust, (Wall, 1980; Welch *et al.*, 1982); however, the incidence of lung cancers has also been observed among workers exposed primarily to arsenate (Bulbulian *et al.*, 1996). The latter study also reported an increased risk of stomach cancers among workers who had been exposed to the highest concentrations of arsenic.

Quantitative dose-response data obtained from copper smelters provide the most compelling evidence that arsenic is responsible for the development of lung cancer (Mazumdar *et al.*, 1989). The conclusions of these studies are nonetheless limited by the confounding exposure to other chemicals, such as sulfur dioxide, and those from cigarette smoking.

An interesting link between arsenic exposure and smoking has been found in a nested case-control analysis of 102 lung cancer cases along with 190 controls. It was found that the incidence of lung cancer increases with increasing arsenic exposure in both smokers and nonsmokers (Järup and Pershagen, 1991). Histological examinations found an increase in several types of lung tumours, indicating that arsenic does not specifically increase the incidence of one particular type of lung cancer.

In addition to lung cancer, other minor types of nonrespiratory cancers associated with inhalation exposure to inorganic arsenic have been reported. Enterline and coworkers (Enterline *et al.*, 1995) found a significantly increased mortality due to cancer of the large intestine and bone cancer. It should be noted, however, that the apparent increase in the risk of bone cancer was based on a very small number of observations.

Other studies have shown an increase in nonmelanoma skin cancers as a result of exposure from a Slovakian coal-burning

power plant (Pesch *et al.*, 2002) and an increase in the risk of stomach cancer among workers exposed to the highest average arsenic concentrations at a Russian fertilizer plant (Bulbulyan *et al.*, 1996).

Human exposure to inorganic arsenic is associated with an increased risk of dermal malignancies (Pi *et al.*, 2008); however, arsenic has been found to act as a cofactor in the development of skin tumours in combination with ultraviolet (UV) irradiation or exposure to phorbol esters. This suggests that the events associated with arsenic-induced dermal carcinogenesis may be distinct from other target tissues.

Long-term arsenic exposure has been reported to cause a malignant transformation of human keratinocytes *in vitro* (Pi *et al.*, 2008). Arsenic-transformed cells were found to show a weakened Nrf2 (nuclear factor E2-related factor 2)-mediated antioxidant defence, activation coupled with apoptotic resistance, increased expression of casein kinase 2 (CK2) and elevated basal Nrf2 activity. Arsenic-induced apoptotic resistance and weakened antioxidant response may therefore be critical steps in development of dermal cancer after exposure to arsenic.

It is generally accepted that methylated organic arsenicals are significantly less toxic than the inorganic forms (Kitchin, 2001). Methylation is part of a natural process of enhanced excretion of arsenic and appears to be a detoxification mechanism for inorganic arsenic. However, the process of methylation may lead to formation of reactive and carcinogenic trivalent methylated arsenicals (MMA(III) and DMA(III); see above) (Cohen *et al.*, 2001, 2002). Thus the process of methylation of inorganic arsenic may provide a toxic pathway and both trivalent methylated arsenic (monomethylarsonous and dimethylarsinous acids) may possess harmful biological activity.

Animal studies did not show any signs of a prospective carcinogenic effect of MMA(V) (reviewed in Cohen *et al.*, 2006). The absence of carcinogenic effect of MMA(V) is in agreement with the negligible amount of DMA(V) formed from exogenously administered MMA(V) (Hughes and Kenyon, 1998).

In contrast to MMA(V), DMA(V) has been shown to induce bladder tumours in rats administered at high doses (100 pm) in the diet for 2 years (Gemert and Eldan, 1998). Since DMA(V) and MMA(V) are stored in the lumen of the bladder, this organ is much more prone to carcinogenic transformation than liver and kidney because of the reductive process leading to formation of DMA(III) and MMA(III) (Kitchin, 2001).

DMA(V) has been documented by a number of studies to act as a cancer promoter in co-administration of other tumorigenic compounds (Wanibuchi *et al.*, 2004; Kitchin, 2001). DMA(V) significantly increased the incidence of bladder, kidney, thyroid gland and liver cancer (Yamamoto *et al.*, 1995). DMA(V) has been reported to act as a skin tumour promoter in mice, accelerating the induction of 7,12-dimethylbenz[a]anthracene (DMBA)-induced skin tumours in mice (Morikawa *et al.*, 2000).

Rats fed with DMA(V) (200 ppm in water) exhibited an increased urinary concentration of DMA(III) in a dose-dependent manner (Okina *et al.*, 2004). This study has also shown that the levels of MMA(III) and DMA(III) may play a significant role in the toxicity and carcinogenicity towards the bladder induced by DMA(V).

It has been proposed that DMA(III) is an unstable metabolite and is stabilized through the formation of a DMA(III)-GSH conjugate, which is responsible for the toxic effect of DMA(III) (Styblo *et al.*, 2000). The cytotoxicity of trivalent (MMA(III), DMA(III) and also of As(III) arsenic can be suppressed by the

application of antioxidants. Positive effects have been found following application of vitamin C and N-acetylcysteine, which preferentially interacts with trivalent arsenicals via its sulfhydryl group (Wei *et al.*, 2005). Interestingly, melatonin and trolox did not show a protective effect against arsenic toxicity. The mechanism of genotoxicity of the DMA(III) does not involve direct interaction with DNA, but is most probably achieved indirectly via formation of ROS (Kitchin and Ahmad, 2003). ROS formation activates the transcription factors (e.g. AP-1, c-fos and NF-kB), and oversecretion of proinflammatory and growth promoting cytokines, resulting in increased cell proliferation and ultimately carcinogenesis.

The exact molecular mechanism of carcinogenesis caused by arsenic is still under investigation by many researchers. Currently accepted molecular mechanisms of arsenic toxicity involve genetic and epigenetic changes, the role of oxidative stress, enhanced cell proliferation and modulation of gene expression. Arsenic is known to induce the hypoxia signalling pathway (Galanis *et al.*, 2009). Treatment of DU145 prostate cancer cells with arsenite induced HIF-1 $\alpha$  expression in a concentration- and time-dependent manner, whereas the level of HIF-1 $\beta$  remained unaffected. The VEGF (vascular endothelial growth factor) protein level was also elevated. ROS formation was linked with the activation of the PI3K/Akt pathway and the subsequent induction of HIF-1 $\alpha$  and VEGF.

### Cardiovascular Effect

While serious and adverse effects on the cardiovascular system following oral exposure to arsenic are well known, there is some evidence from epidemiological studies that the cardiovascular system may also be affected by inhaled inorganic arsenic (Navas-Acien *et al.*, 2005; States *et al.*, 2009).

Among the more profound effects on the heart from long-term exposure to arsenic are altered myocardial depolarization and cardiac arrhythmias (Cullen *et al.*, 1995; Mumford *et al.*, 2007). Long-term, low/medium-level exposure has been shown to cause mild damage to the vascular system; however, severe hypertrophy of the ventricular wall was observed after an acute exposure to a high (93 mg) concentration of arsenic (Quatrehomme *et al.*, 1992).

Wang and coworkers (Wang *et al.*, 2003) found an increased incidence of disease in the blood vessels in Taiwanese populations living in areas with arsenic-polluted wells ( $>0.35 \text{ mg l}^{-1}$ ). In addition, attempts to assess the relative risks for stroke and peripheral arterial disease have been conducted. However, there are methodologic limitations for the interpretation of the observed data and it would hence appear sensible to make such studies of the effect of arsenic on the cardiovascular system a research priority.

In another ecological study conducted in the USA, a significant increase in the number of deaths from arteriosclerosis, aneurysm and other related diseases were found in the areas in which the drinking water contained arsenic concentrations  $>20 \text{ } \mu\text{g l}^{-1}$  (Engel and Smith, 1994). No significant cardiovascular effects were noted after acute ingestion of monosodium methylarsenate ( $1714 \text{ mg kg}^{-1}$ ).

Vascular endothelium is well known to regulate the release of various mediators such as nitric oxide, angiotensin-II, endothelin-1, adhesion molecules, cytokines and other similarly acting species (Balakumar and Kaur, 2009; Quyyumi, 1998). NO has been considered to be a major mediator released from endothelium. It has vasodilatory and anti-inflammatory

properties, and inhibits platelet adhesion and aggregation, smooth muscle cell proliferation and migration. Exposure of endothelial cells to sodium arsenite induces a decline in the integrity of vascular endothelium and endothelial cytotoxicity by inactivating protein kinase B/Akt and eNOS, so reducing the generation and bioavailability of NO, and increasing the oxidative stress and subsequently decreasing the endothelium-dependent vasorelaxation (Balakumar and Kaur, 2009).

Arsenic has been shown to induce atherosclerosis by increasing mRNA transcripts of growth factors including granulocyte-macrophage colony-stimulating factor, transforming growth factor- $\alpha$  and the inflammatory cytokinelike tumour necrosis factor- $\alpha$  (Germolec et al., 1997; Kitchin, 2001). Experimental studies of the effect of arsenic on the vascular system have shown that oxidized lipids are present in all stages of atherogenesis which in turn generate several bioactive molecules (e.g. ROS, peroxides and isoprostanes), of which aldehydes are the major end products. Malondialdehyde (MDA) and 4-hydroxy-trans-2-nonenal (HNE) are the most abundant aldehydes generated from the oxidation of LDL and possess mutagenic and carcinogenic properties (Valiko et al., 2005, 2007). Protein adducts of MDA and HNE have been detected in atherosclerotic lesions of experimental animals and humans.

Evidence from a large number of studies indicates that inflammation plays a pivotal role in atherosclerotic plaque formation. Vascular cells generate chemokines and proinflammatory cytokines including monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and tumour necrosis factor  $\alpha$ . This suggests that As-induced inflammation could be an important risk factor for atherosclerosis (Tsou et al., 2005).

Hypertension is another disorder associated with increased arsenic exposure (Yang et al., 2007). Arsenic-induced hypertension has been explained by an enhanced myosin light-chain phosphorylation and an increase in calcium-sensitization in blood vessels. Disruption of the antioxidant defence system leads to elevated systolic blood pressure. The possible mechanisms of arsenic-induced atherosclerosis, vascular endothelial dysfunction and hypertension are shown in Fig. 4.

## Gastrointestinal Disturbances

### Inorganic arsenicals

Clinical signs of gastrointestinal irritation, including nausea, vomiting, diarrhoea and abdominal pain, are observed in all cases of short-term high-dose and longer-term lower-dose exposures to inorganic arsenic (Ueda and Furukawa, 2003; Vantroyen et al., 2004). Haemorrhagic gastrointestinal lesions have been reported in animal studies. For example, a monkey fed with 6 mg As kg<sup>-1</sup> per day for approximately 1 month was found, upon necropsy, to have died of acute inflammation and haemorrhage of the small intestine (Heywood and Sortwell, 1979).

### Organic arsenicals

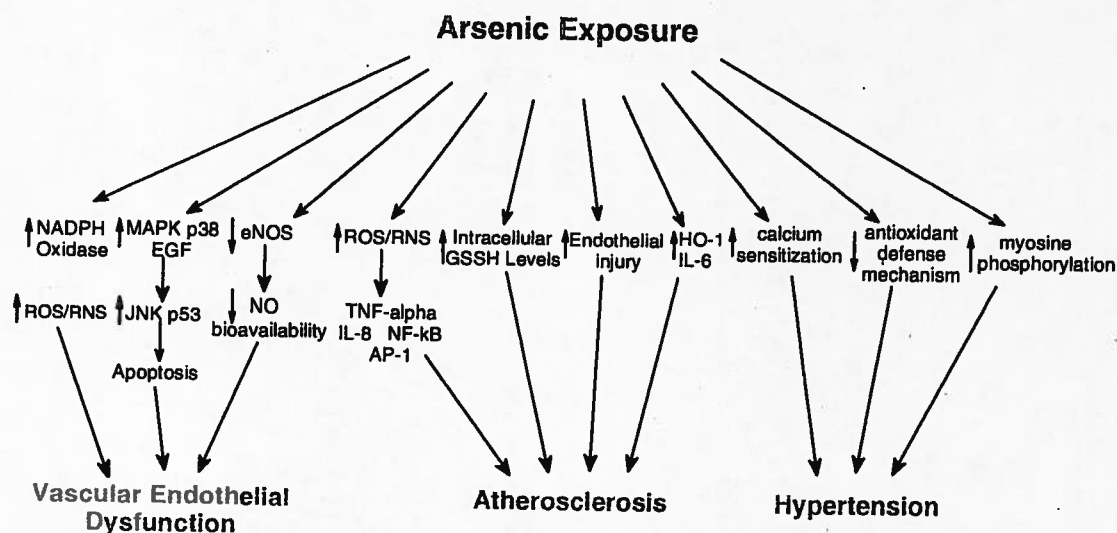
The gastrointestinal tract appears to be the critical target of toxicity following oral exposure to MMA. Ingestion of 80 mg kg<sup>-1</sup> of organic arsenicals causes vomiting, abdominal pain, hyperactive bowel and diarrhoea (Lee et al., 1995).

A dose level of 72.4 mg MMA kg<sup>-1</sup> per day led to a thickened wall, oedema and haemorrhagic, necrotic, ulcerated or perforated mucosa in the large intestine and a significant increase in the incidence of squamous metaplasia of the epithelial columnar absorptive cells in the colon and rectum. Squamous metaplasia was also observed in the colon of mice chronically exposed to 67 mg MMA kg<sup>-1</sup> per day (Arnold et al., 2003; Gur et al., 1991).

## Liver Disease

### Inorganic arsenicals

A number of studies revealed symptoms of hepatic injury after oral exposure of humans to inorganic arsenic. These effects were most frequently observed after repeated exposure to doses of 0.01–0.1 mg As kg<sup>-1</sup> per day. Clinical examination confirmed liver damage (Liu et al., 2002) and blood tests showed elevated levels of hepatic enzymes. Histological examination of the livers has revealed a consistent finding of portal tract fibrosis (Mazumder et al., 2005). Individuals exposed more frequently to arsenic suffered from cirrhosis, which was considered to be a secondary effect of damage to the hepatic blood vessels.



**Figure 4.** The influence of arsenic on the mechanisms of the vascular endothelial dysfunction, atherosclerosis and hypertension.

Increased concentrations of zinc and copper were detected in the livers of rats receiving a single oral dose of 10 mg As kg<sup>-1</sup> as sodium arsenite (Flora and Tripathi, 1998). Hepatic levels of malondialdehyde and glutathione were decreased in the livers of rats receiving 200 mg As kg<sup>-1</sup> as GaAs (Flora *et al.*, 1998). An increase in peroxidation markers was reported in rats administered with 0.02 mg As kg<sup>-1</sup> per day for 60 days from drinking water containing 2.5 mg sodium arsenite l<sup>-1</sup> (Bashir *et al.*, 2006).

#### Organic arsenicals

No studies of the hepatic effects of organic arsenicals on humans have been reported. Histology of livers from rabbits repeatedly given MMA showed diffuse inflammation and mild hepatocellular degeneration (Jaghabir *et al.*, 1989). Rats exposed to a dose of 72.4 mg MMA kg<sup>-1</sup> per day for 104 weeks showed a decrease in absolute liver weight. While rats exposed to DMA (Siewicki, 1981) did not exhibit any effect, mice exposed to oral doses of 720 mg DMA kg<sup>-1</sup> exhibited decreased liver glutathione and cytochrome P-450 content and reduced serum ornithine decarboxylase activity (Ahmad *et al.*, 1999).

#### Renal Disease

Inorganic arsenicals do not cause any significant renal injury in humans. In some cases elevated levels of creatinine or bilirubin have been reported (Moore *et al.*, 1994). Similarly, animal studies indicated that the kidney is not a major target for inorganic arsenic. However, at high levels of exposure, mild histological changes in the renal tubules of monkeys have been noted. Animal studies have reported renal and urinary bladder effects following oral exposure to organic arsenicals. The urinary system is a more sensitive target for DMA than for MMA (Cohen *et al.*, 2001). Animal experiments have shown that DMA induced renal damage characterized by increased volume and pH of urine and decreased electrolyte levels, increased urinary calcium levels and an increase in water consumption. In addition, increased kidney weights and minimal tubular epithelial cell degeneration, tubular casts and focal mineralization were observed. Some studies on rats exposed to DMA have reported damage to the urinary bladder. The observed damage included altered bladder cell surfaces.

#### Neurological Disorders

##### Inorganic arsenicals

Inorganic arsenic can cause serious neurological effects, after both inhalation (Calderon *et al.*, 2001; Lagerkvist and Zetterlund, 1994) and oral exposure (Uede and Furukawa, 2003). This conclusion is based mainly on clinical observations and neurological examinations of exposed individuals.

Animal studies have shown that neurological effects following rat exposure to arsenic in the form of sodium arsenite involve changes in levels of neurotransmitters such as dopamine, norepinephrine and 5-hydroxytryptamine (Kannan *et al.*, 2001). Since adult animals appear to be much less susceptible to the neurological effects of inorganic arsenic than humans, studies in adult animals would probably not help to estimate a safe human exposure limit.

Recent findings indicate a possible association between arsenic in drinking water and neurobehavioral alterations in children (Tsai *et al.*, 2003). Adolescents from various regions of

Taiwan and China exposed to low (0.0017–0.0018 mg As kg<sup>-1</sup> per day) levels of inorganic arsenic in their drinking water showed a decreased performance in the switching attention task, while children in the high exposure group (0.0034–0.0042 mg As kg<sup>-1</sup> per day) showed a decreased performance in both the switching attention task and in tests of pattern memory, relative to unexposed controls.

Ingestion of inorganic arsenic can cause injury to the nervous system. Exposure at a level of 2 mg As kg<sup>-1</sup> per day or more can lead to encephalopathy, with symptoms of headache, mental confusion, seizures and coma (Bartolome *et al.*, 1999). Prolonged exposures to lower levels of arsenic (0.03–0.1 mg As kg<sup>-1</sup> per day) are typically characterized by a symmetrical peripheral neuropathy (Chakraborti *et al.*, 2003; Foy *et al.*, 1992), which in its early stages is characterized by numbness in the hands and feet then further develops into a painful pins-and-needles sensation. Both sensory and motor nerves are affected and muscle weakness often develops (Goebel *et al.*, 1990). A certain degree of recovery is possible once the subject has been removed from the contaminated area, but most commonly this is only partial (Fincher and Koerker, 1987).

The most typical neurological feature of arsenic neurotoxicity is peripheral neuropathy which may last for several years (Mathew *et al.*, 2010). Studies on patients with As neuropathy have shown a reduced nerve conducting velocity in their peripheral nerves, and this has become a hallmark of As-induced neurotoxicity, as is a typical feature of axonal degeneration. The majority of the unfavourable effects of Arsenic are caused by the inactivation of enzymes that are important for cellular energy metabolism, whereby As reacts with the thiol groups of proteins and enzymes and inhibits their catalytic activity. In a similar fashion to other neurodegenerative diseases, arsenic-induced neurotoxicity causes changes in cytoskeletal protein composition and hyperphosphorylation. These changes may lead to disorganization of the cytoskeletal structure, which is a potential cause of As-induced neurotoxicity.

##### Organic arsenicals

No neurological symptoms or brain lesions were observed following chronic exposure of rats to MMA (72.4 mg kg<sup>-1</sup> per day) or mice to MMA kg<sup>-1</sup> per day (67.1 mg; Arnold *et al.*, 2003). Two further studies in pigs indicated that oral doses of roxarsone can cause significant neurotoxicity in which the main feature is a time-dependent degeneration of myelin and axons (Kennedy *et al.*, 1986).

#### Reproductive Health Effects

Animal studies have shown that reproductive activity was unaffected in rats receiving doses of 8 mg of As<sub>2</sub>O<sub>3</sub> from 14 days prior to mating. The evaluation of reproductive activity included a mating index, a fertility index and the precoital interval (time before mating) index (Holson *et al.*, 1999).

A more comprehensive, three-generation study of arsenic intake in drinking water in mice revealed a significant increase in the incidence of small litters and a trend toward a smaller number of pups per litter in all three generations of the treated group (Schroeder and Mitchener, 1971).

This conclusion was recently confirmed by another study which showed changes in several reproductive system end points, including reduced weights of the uterus and ovary and reduced ovarian and uterine peroxidase activities; inhibition of

steroidogenic enzymes and decreased estradiol levels relative to the controls (Chattopadhyay et al., 2001, 2003).

### Antioxidant Protection Against Arsenic Mutagenicity

Oxidative stress to DNA is recognized as an underpinning component of the mechanism of arsenic carcinogenesis (Valiko et al., 2005). Antioxidant enzymes are considered to be the first line of cellular defence against oxidative damage. Superoxide dismutase (SOD) and catalase (CAT) are the most important, first line antioxidant defence in cells exposed to oxygen. SOD catalyses the dismutation of superoxide into oxygen and hydrogen peroxide, while CAT catalyses the decomposition of hydrogen peroxide to water and oxygen. Arsenic-intoxicated rats revealed reduced activity of SOD which was attributed to the enhanced production of superoxide radical anions.

A second line of cellular defence system against free radical-induced damage is provided by a thiol-based antioxidant system (Manna et al., 2008). Decreased GSH pools and increased levels of lipid peroxidation due to arsenic toxicity were found to lead to a decrease in the activities of GST and GPx with a concomitant decrease in the activity of the GSH-regenerating enzyme GR.

A field trial was undertaken in West Bengal (a region whose population is exposed to high levels of arsenic in drinking water), to evaluate the role of the phytochemical, curcumin, from turmeric for its antioxidant and antimutagenic activity (Biswas et al., 2010). Blood samples taken from volunteers in the region showed notable DNA damage and depleted antioxidant activity. However, following dosage with curcumin capsules for 3 months, the DNA damage was reduced, ROS generation and lipid peroxidation were suppressed, and the antioxidant activity of blood plasma was raised, thus offering the hope of some protective role for curcumin against DNA damage by arsenic.

The most effective known treatment for arsenic poisoning is chelation therapy; however such agents as British anti lewisite, sodium 2,3-dimercaptopropane-1-sulfonate, meso 2,3-dimercaptosuccinic acid etc. result in a number of undesirable side-effects (Flora et al., 2007). It has been shown that supplementation of the chelating agent with antioxidants may be beneficial in achieving optimum effects.

Another study reported genotoxic effects of sodium arsenite (known for its genotoxic effects through ROS generation) in forming micronuclei in the polychromatic erythrocytes in the bone marrow cells of Wistar rats (Balakumar et al., 2010). Supplementation by orally administered  $\alpha$ -tocopherol (400 mg kg<sup>-1</sup> of body weight) and ascorbic acid (200 mg kg<sup>-1</sup> of body weight) to rats given 100 ppm of sodium arsenite in their drinking water for 30 days suggested a protective effect on the cellular antioxidant system and a modulation of arsenic-induced micronuclei formation.

### CONCLUSION

Arsenic exposure affects millions of people worldwide. Epidemiological studies appear to provide an important guide for arsenic risk assessment in water, air or dust. Research work on arsenic poisoning has revealed that free radical-mediated oxidative damage is a common denominator of arsenic pathogenesis. A dose-dependent relationship between arsenic concentration and cancer incidence has been found, however, only among highly exposed populations.

Although arsenic-induced formation of various cancers has been widely studied, less attention has been paid to arsenic-induced cardiovascular disorders, even though epidemiological studies have shown that chronic arsenic exposure is associated with increased morbidity and mortality from cardiovascular disease. Arsenic has been found to initiate endothelial dysfunction by diminishing the integrity of vascular endothelium followed by inactivation of the eNOS, which therefore reduces the generation and bioavailability of nitric oxide and increases oxidative stress.

Arsenic-induced formation of ROS and subsequent depletion of antioxidant cell defences can result in disruption of the antioxidant/prooxidant equilibrium in mammalian tissues. Owing to its sulfhydryl group binding capacity, arsenic can also inhibit the activities of many enzymes, especially those involved in the uptake of glucose in cells, fatty acid oxidation and production of glutathione.

Although the toxic and carcinogenic effects on humans exposed to arsenic have been well documented, the mechanisms by which arsenic induces health effects, including cancer, cardiovascular disorders, metabolic disease and other diseases are not well characterized. To provide a deeper understanding of the pathology of arsenic-induced diseases and the toxicology of arsenic in various organs, further research is necessary.

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## ORIGINAL RESEARCH COMMUNICATION

# Tanshinone I Activates the Nrf2-Dependent Antioxidant Response and Protects Against As(III)-Induced Lung Inflammation *In Vitro* and *In Vivo*

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### Abstract

**Aims:** The NF-E2 p45-related factor 2 (Nrf2) signaling pathway regulates the cellular antioxidant response and activation of Nrf2 has recently been shown to limit tissue damage from exposure to environmental toxicants, including As(III). In an attempt to identify improved molecular agents for systemic protection against environmental insults, we have focused on the identification of novel medicinal plant-derived Nrf2 activators. **Results:** Tanshinones [tanshinone I (T-I), tanshinone IIA, dihydrotanshinone, cryptotanshinone], phenanthrenequinone-based redox therapeutics derived from the medicinal herb *Salvia miltiorrhiza*, have been tested as experimental therapeutics for Nrf2-dependent cytoprotection. Using a dual luciferase reporter assay overexpressing wild-type or mutant Kelch-like ECH-associated protein-1 (Keap1), we demonstrate that T-I is a potent Keap1-C151-dependent Nrf2 activator that stabilizes Nrf2 by hindering its ubiquitination. In human bronchial epithelial cells exposed to As(III), T-I displays pronounced cytoprotective activity with upregulation of Nrf2-orchestrated gene expression. In Nrf2 wild-type mice, systemic administration of T-I attenuates As(III) induced inflammatory lung damage, a protective effect not observed in Nrf2 knockout mice. **Innovation:** Tanshinones have been identified as a novel class of Nrf2-inducers for antioxidant tissue protection in an *in vivo* As(III) inhalation model, that is relevant to low doses of environmental exposure. **Conclusion:** T-I represents a prototype Nrf2-activator that displays cytoprotective activity upon systemic administration targeting lung damage originating from environmental insults. T-I based Nrf2-directed systemic intervention may provide therapeutic benefit in protecting other organs against environmental insults. *Antioxid. Redox Signal.* 00, 000–000.

### Introduction

INORGANIC As(III) is a ubiquitous environmental contaminant found in water and dust that poses a considerable threat to human health worldwide (2, 3, 5, 6). Among various organ systems, the lung has been identified as a major target organ for As(III)-induced acute and chronic toxicities (8, 9, 16, 22, 40). Indeed, a strong association between As(III) exposure and increased incidence of pulmonary malfunctions (such as chronic cough, bronchitis and shortness of breath) has been substantiated, and human epidemiological studies provide strong evidence in support of a carcinogenic

### Innovation

Tanshinones have been identified as a novel class of NF-E2 p45-related factor 2 (Nrf2)-inducers for antioxidant tissue protection in an *in vivo* As(III) inhalation model, that is relevant to low doses of environmental exposure.

activity of As(III) on the lung (38, 40, 41, 49). Therefore, an urgent need exists for the identification and development of protective strategies that limit As(III)-induced lung damage.

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Cumulative evidence suggests that the NF-E2 p45-related factor 2 (Nrf2) signaling pathway represents an important cellular defense system that suppresses tissue damage caused by acute or chronic exposure to various environmental toxicants, including solar ultraviolet radiation, xenobiotics, and heavy metals (10, 20, 42, 59). Enhanced sensitivity of Nrf2 knockout (Nrf2<sup>-/-</sup>) versus Nrf2 wild-type (Nrf2<sup>+/+</sup>) mice towards pulmonary damage from various toxicological challenges, including diesel exhaust particles, cigarette smoke, diquat, and As(III) has been documented (4, 24, 26, 43, 57). Our earlier experiments have demonstrated that stable knockdown of endogenous Nrf2 rendered cells more sensitive to As(III)-induced cell death (53). We and others have also demonstrated the interventional potential of diet-derived Nrf2 activators including sulforaphane (SF), lipoic acid, and cinnamaldehyde for the suppression of As(III) cytotoxicity through modulation of the Nrf2-dependent cellular defense mechanism (15, 23, 48, 53, 56). For example, SF has been shown to limit organ toxicity of As(III) *in vivo* when As(III) was administered through drinking water (25). More recently, we have shown that Nrf2 status determines the sensitivity of mice to As(III)-induced lung damage and that Nrf2 modulation using SF prevents inflammatory tissue damage in an *in vivo* As(III) inhalation model that is relevant to low environmental human exposure to As(III)-containing dusts (62).

The transcription factor Nrf2 is thought to control tissue damage from environmental insults by upregulating the expression of genes involved in antioxidant response and xenobiotic metabolism (20, 25, 26, 59). Nrf2 is negatively regulated by Kelch-like ECH-associated protein-1 (Keap1), which forms an E3 ubiquitin ligase complex with Cullin 3 (Cul3) and Rbx1 (61). Under normal conditions, cells maintain low constitutive levels of Nrf2 because the Keap1-E3 ubiquitin ligase complex constantly ubiquitinates Nrf2 and targets it for degradation by the 26S proteasome. When cells are exposed to exogenous stimulants, such as SF and tert-butylhydroquinone (tBHQ), the activity of the Keap1-E3 ubiquitin ligase complex is thought to be impaired due to modifications of critical cysteine residues in Keap1, particularly C151, leading to stabilization of Nrf2 (60). Nrf2 then translocates to the nucleus and binds to the antioxidant response element (ARE) in the promoter regions of cytoprotective genes (13, 17, 27, 50, 61). The Nrf2-target genes encode proteins with diverse cellular functions. For example,  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS) and NAD(P)H quinone oxidoreductase (NQO1) are involved in the synthesis of glutathione (GSH) and redox homeostasis, respectively (7, 45, 46, 55). Conjugating enzymes, including glutathione S-transferases (GSTs) and UDP-glucuronosyltransferase, facilitate the removal of toxic and carcinogenic chemicals by increasing their solubility and excretion (19, 21, 28). Transporters, such as multidrug resistance proteins and p-glycoproteins, are important in the uptake and removal of xenobiotics (18, 37, 51, 58).

Interestingly, the "dark" side of Nrf2 has been recently revealed. Somatic mutations in either Keap1 or Nrf2 that disrupt Keap1-mediated negative regulation of Nrf2 have been identified in many types of cancer, which result in high constitutive levels of Nrf2. In addition, reduced expression of Keap1, due to hypermethylation or loss of heterozygosity, also leads to high basal levels of Nrf2 in certain cancers (32, 44). As(III) itself is an Nrf2 activator and has been shown to

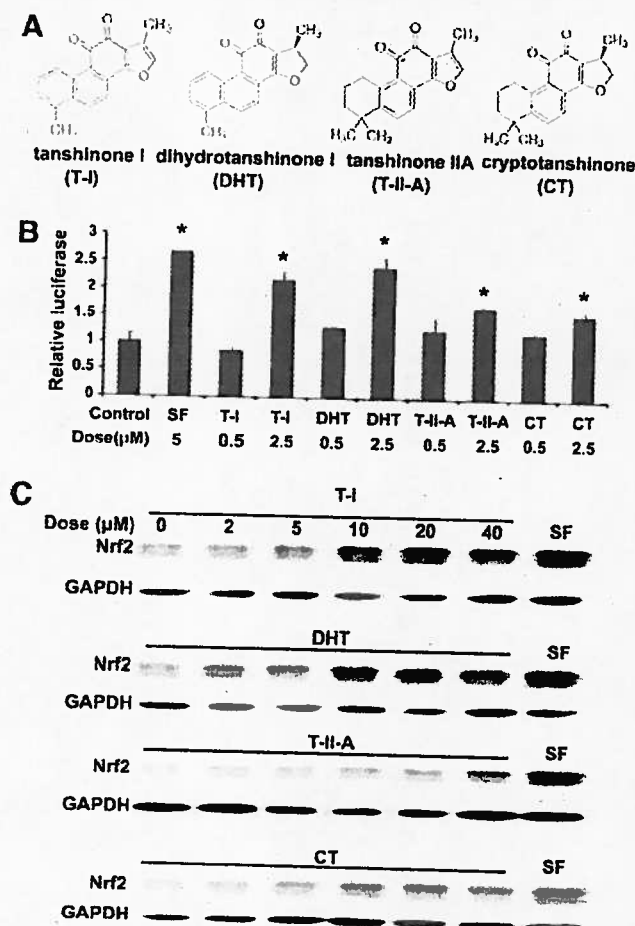
induce the Nrf2 pathway in many cell lines *in vitro* and in the liver, bladder, and lung when As(III) was administered through drinking water or inhaled particles *in vivo* (25, 33, 62). It is perplexing how could both beneficial chemopreventive compounds and harmful arsenicals induce Nrf2. Intriguingly, in our recent studies we uncovered a distinct mechanism of Nrf2 activation by As(III) at low environmentally relevant doses that is different from that of dietary chemopreventive compounds, such as SF and tBHQ (33, 54) (manuscript accepted to *Molecular and Cellular Biology*). Unlike SF and tBHQ that activate Nrf2 in a Keap1-C151 dependent, and p62-independent manner (canonical), As(III) activates Nrf2 in a noncanonical manner that is Keap1-C151 independent, and p62-dependent. Detailed studies indicate that As(III) causes an increase of autophagosomes where Keap1 and p62 accumulates, leading to inactivation of Keap1 and upregulation of Nrf2. More importantly, the noncanonical activation of Nrf2 by arsenic results in a prolonged activation of Nrf2, which mimics high constitutive levels of Nrf2 observed in certain cancer types, deemed the dark side of Nrf2 (33) (manuscript accepted to *Molecular and Cellular Biology*). It is likely that the prolonged activation of Nrf2 by arsenic underlies arsenic toxicity and carcinogenicity.

Elucidating the differences in the mode of Nrf2 activation between canonical versus noncanonical Nrf2 activators led us to hypothesize that Nrf2 activation by canonical Nrf2 activators is protective, whereas its activation by noncanonical Nrf2 activators is harmful. In addition, the canonical Nrf2 activators can alleviate arsenic-mediated toxic effects. In an attempt to identify improved canonical Nrf2 activators for systemic protection against As(III)-induced tissue damage we have focused on medicinal plant-derived Nrf2 activators. Here we demonstrate that tanshinone I (T-I), a phenanthrenequinone constituent of the Chinese medicinal herb Danshen (*Salvia miltiorrhiza*), is a potent Keap1-C151-dependent Nrf2 activator that stabilizes Nrf2 by hindering its ubiquitination. In human bronchial epithelial (HBE) cells exposed to As(III), T-I displayed pronounced cytoprotective activity with upregulation of Nrf2-orchestrated gene expression. In an *in vivo* As(III) inhalation model, T-I activated the Nrf2 signaling pathway attenuating As(III)-induced inflammation in lungs from Nrf2<sup>+/+</sup> but not Nrf2<sup>-/-</sup> mice.

## Results

### Identification of tanshinones I and dihydrotanshinone as Nrf2-inducers

Employing a dual luciferase reporter assay with the mGST-ARE firefly luciferase plasmid reported previously, we measured the ability of four major tanshinones [T-I, dihydrotanshinone (DHT), tanshinone IIA (T-IIA), and cryptotanshinone (CT)] in inducing the transcriptional activity of Nrf2 (Fig. 1A). The transcriptional activity of Nrf2 was upregulated upon exposure to low micromolar concentrations that was most pronounced in response to T-I and DHT (Fig. 1B). Similarly, the protein level of Nrf2 was enhanced by tanshinone exposure (Fig. 1C). Again, T-I and DHT were the most active tanshinones causing upregulation of Nrf2 at the protein level similar to that induced by SF (Fig. 1C). Based on the activity of T-I in upregulating Nrf2 we focused our subsequent mechanistic and cytoprotection studies on T-I.



**FIG. 1.** Nrf2 activation by tanshinones. (A) Chemical structures of T-I, DHT, T-II-A, and CT. (B) MDA-MB-231 cells transfected with expression vectors for mGST-ARE firefly luciferase and renilla luciferase reporters were left untreated or treated with each of the indicated compounds for 16 h. Dual luciferase activities were measured. The experiment was repeated three times, with triplicate samples in each experiment. The data are expressed as means  $\pm$  SD (\* $p$  < 0.05). (C) MDA-MB-231 cells were exposed to the indicated doses of tanshinones for 4 h. SF (5  $\mu$ M) treatment for 4 h was included as a positive control. Total cell lysates were subjected to immunoblot analysis. ARE, antioxidant response element; CT, cryptotanshinone; DHT, dihydrotanshinone; GST, glutathione S-transferase; Nrf2, NF-E2 p45-related factor 2; SF, sulforaphane; T-I, tanshinone I; T-II-A, tanshinone IIA; SD, standard deviation.

#### T-I activates Nrf2 in a C151-dependent manner and decreases its ubiquitination

Consistent with the result demonstrating upregulation of Nrf2 at the protein level in response to T-I treatment (Fig. 1C), the transcriptional activity of Nrf2 is also induced by T-I in a dose-dependent manner (Fig. 2A). At doses  $\geq 4 \mu$ M a significant induction of luciferase activity was observed that was five fold over control at 20  $\mu$ M, at which dose there was no observable toxicity (Fig. 2A).

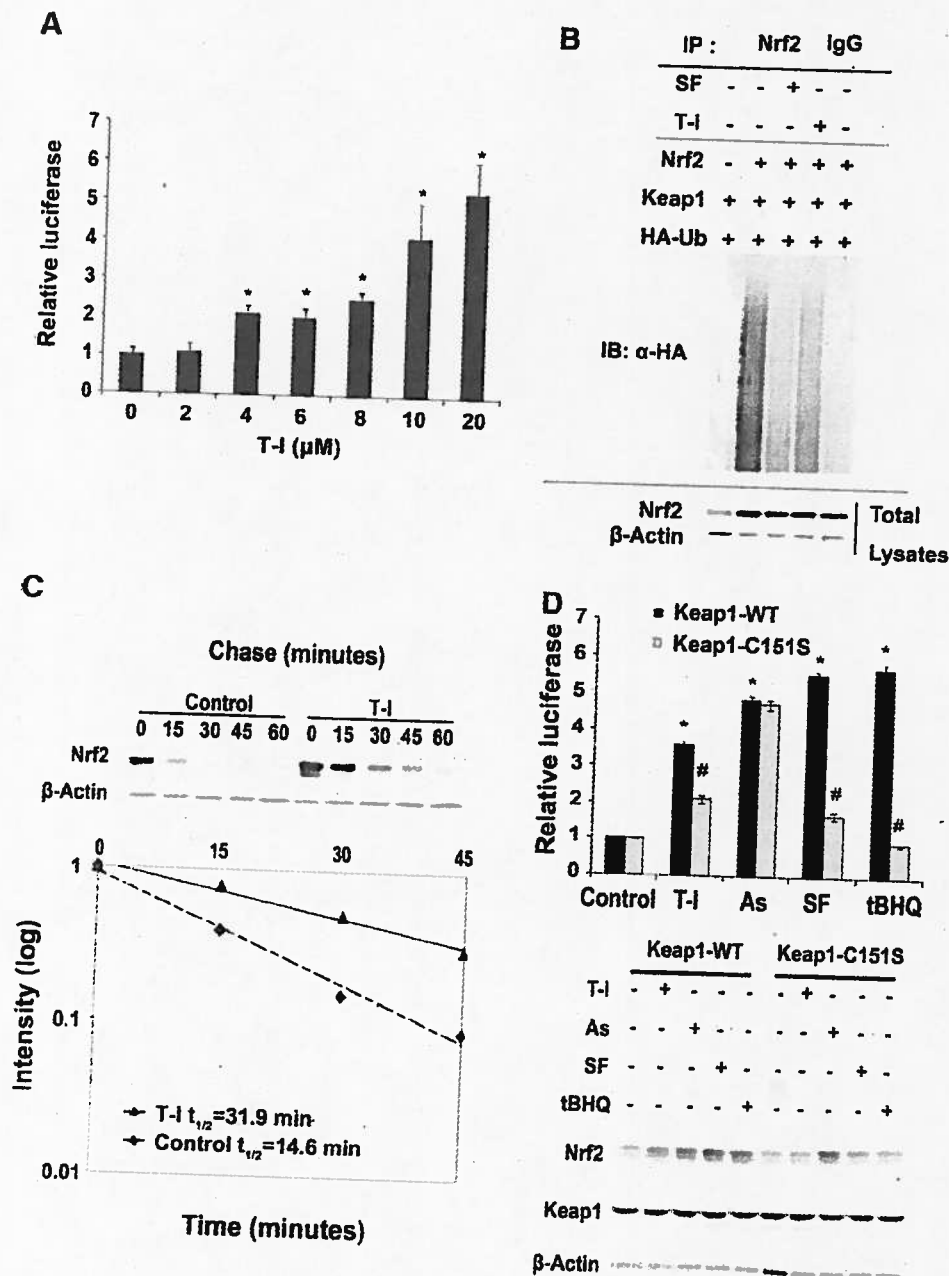
Next, the mechanism of Nrf2 activation by T-I was investigated. As demonstrated previously, established Nrf2 inducers, such as SF and tBHQ cause Nrf2 activation through

inhibition of the Keap1-mediated ubiquitination of Nrf2 (60). Therefore, an *in vivo* ubiquitination assay was performed using MDA-MB-231 cells cotransfected with expression vectors for Nrf2, Keap1, and HA-ubiquitin, and were either left untreated or treated with SF or T-I. When cells were treated with T-I, ubiquitination of Nrf2 decreased compared to the untreated control (Fig. 2B). As expected, SF also decreased Nrf2 ubiquitination (Fig. 2B). Next, the half-life of endogenous Nrf2 protein was determined. Cycloheximide was added to untreated or T-I-treated cells for the indicated time. The protein level of Nrf2 was detected by immunoblot analysis (Fig. 2C, upper panel). The intensity of the Nrf2 band was measured and plotted against the time after addition of cycloheximide and the half-life of Nrf2 was calculated (Fig. 2C, lower panel). The half-life of Nrf2 under untreated conditions was 14.6 min; however, after T-I treatment, the half-life of Nrf2 increased to 31.9 min (Fig. 2C). These results indicate that T-I activates the Nrf2-dependent response by decreasing its ubiquitination and thus, stabilizing Nrf2 at the protein level.

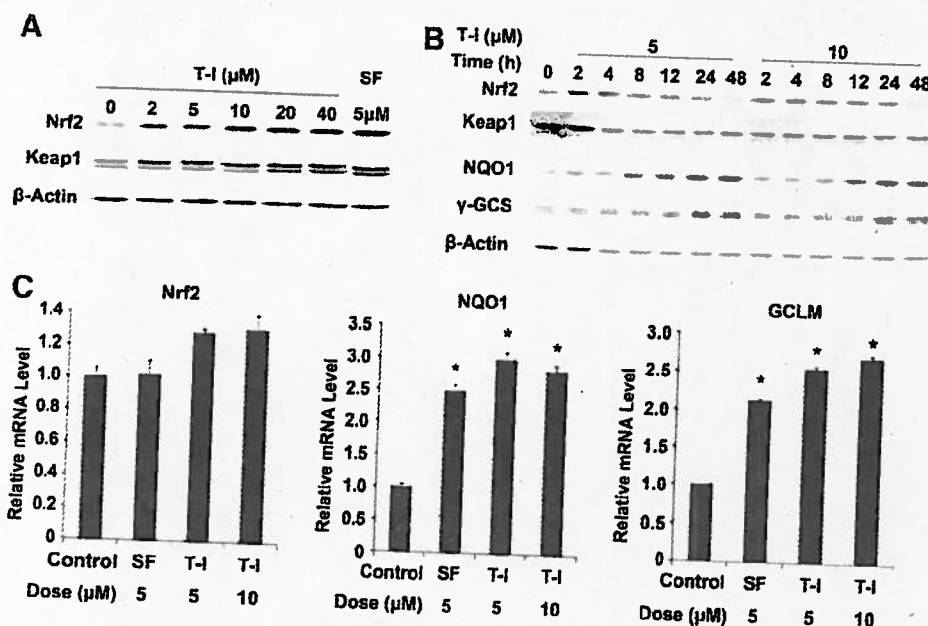
Previously, we reported that the critical cysteine residue at amino acid 151 (C151) in Keap1 is required for activation of Nrf2 by SF or tBHQ. We also have shown that As(III)-induced Nrf2 activation occurs independent of Keap1 (C151) (54, 60). Therefore, to determine the specific mechanism by which T-I activates the Nrf2 pathway, we investigated whether T-I-mediated Nrf2 activation was also dependent on C151 (Fig. 2D). A specific Keap1-siRNA targeting the 3' untranslated region was included during transfection to knockdown the expression of endogenous Keap1. Cells were then cotransfected with expression vectors for either Keap1-WT or Keap1-C151S along with ARE-firefly luciferase and renilla luciferase. Before measurement of dual luciferase, cells were treated with As(III), SF, and tBHQ for 16 h. Pronounced enhancement of Nrf2 activity was observed with all treatments [T-I, As(III), SF, tBHQ] when Keap1 wild-type (Keap1-WT) was cotransfected (Fig. 2D, upper panel; black bars). In contrast, upon expression of the mutated Keap1-C151S, Nrf2 activation by T-I, SF, and tBHQ was dramatically blunted, but As(III) was still able to activate Nrf2 (Fig. 2D, upper panel; gray bars). This is consistent with a C151-dependent (T-I, SF, tBHQ) or independent [As(III)] mechanism of activation. Immunoblot analysis of cell lysates further confirmed the results obtained from the luciferase assay. For cells transfected with Keap1-WT, all treatments increased the protein level of Nrf2 (Fig. 2D, lower panel). When Keap1-C151S was transfected into cells, only As(III) increased Nrf2; however, induction of Nrf2 by T-I, SF, and tBHQ was blocked (Fig. 2D, lower panel). These results demonstrate for the first time that T-I is a canonical Nrf2 inducer and it activates the Nrf2 pathway through the critical C151 sensor residue in Keap1.

#### T-I activates Nrf2 and its downstream genes in HBE cells and protects HBE cells from As(III)-induced oxidative stress and cytotoxicity

Since the lung is the major target organ for arsenic-mediated toxicity and carcinogenicity, the ability of T-I to activate the Nrf2 signaling pathway was tested in HBE cells. When cells were exposed to T-I for 4 h, endogenous Nrf2 protein levels increased in a dose-dependent manner (Fig. 3A). Similar to SF, T-I had no effect on Keap1 protein levels (Fig. 3A). Next, the time dependent induction of Nrf2 by T-I



**FIG. 2.** T-I activates Nrf2 by decreasing Nrf2 ubiquitination and increasing Nrf2 stability in a Keap1-C151-dependent manner. (A) A dual luciferase reporter assay was performed after cotransfected MDA-MB-231 cells were treated with the indicated concentration of T-I for 16 h. The data are expressed as means  $\pm$  SD (\* $p$  < 0.05). (B) MDA-MB-231 cells were cotransfected with the plasmids encoding the indicated proteins. Cells were then treated with either SF (5  $\mu$ M) or T-I (5  $\mu$ M) along with MG132 (10  $\mu$ M) for 4 h before cell lysates were collected for ubiquitination assay. Anti-Nrf2 immunoprecipitates were analyzed by immunoblot with anti-HA antibodies for detection of ubiquitin-conjugated Nrf2. (C) Cells were either left untreated or treated with T-I (5  $\mu$ M) for 4 h. Cycloheximide (50  $\mu$ M) was added and cells were lysed at the indicated time points. Cell lysates were subjected to immunoblot analysis using anti-Nrf2 and anti- $\beta$ -actin antibodies. The intensity of the bands was quantified using Quantity One software and plotted against the time after cycloheximide treatment. (D) The ARE luciferase reporter gene assay was performed in the same way as described in (A), except that Keap1-C151S, rather than Keap1-WT, was cotransfected in half of the samples. In addition, a Keap1-siRNA against the 3' untranslated region was cotransfected to suppress endogenous Keap1. The transfected cells were then treated with T-I (5  $\mu$ M), As(III) (10  $\mu$ M), SF (5  $\mu$ M), or tBHQ (50  $\mu$ M) for 16 h, before the measurement of firefly and renilla luciferase activities. Data were expressed as means  $\pm$  SD (\* $p$  < 0.05 control vs. compound treatment; \* $p$  < 0.05 Keap1-WT vs. Keap1-C151S). An aliquot of cell lysates was used for immunoblot analysis. Keap1, Kelch-like ECH-associated protein-1; tBHQ, tert-butylhydroquinone.



**FIG. 3.** T-I activates the Nrf2 pathway. (A) HBE cells were treated with the indicated doses of T-I for 4 h. Cell lysates were subjected to immunoblot analysis. (B) HBE cells were treated with T-I (5 or 10  $\mu$ M) for the indicated duration. Cell lysates were subjected to immunoblot analysis. SF was included as a positive control in both (A) and (B). (C) HBE cells were treated with T-I (5 or 10  $\mu$ M) for 16 h and mRNA was extracted. The relative mRNA levels of Nrf2, NQO1, and GCLM were then determined by quantitative real-time RT-PCR. Data are expressed as means  $\pm$  SD (\* $p$  < 0.05). HBE, human bronchial epithelial; NQO1, NAD(P)H quinone oxidoreductase; RT-PCR, reverse transcription-polymerase chain reaction.

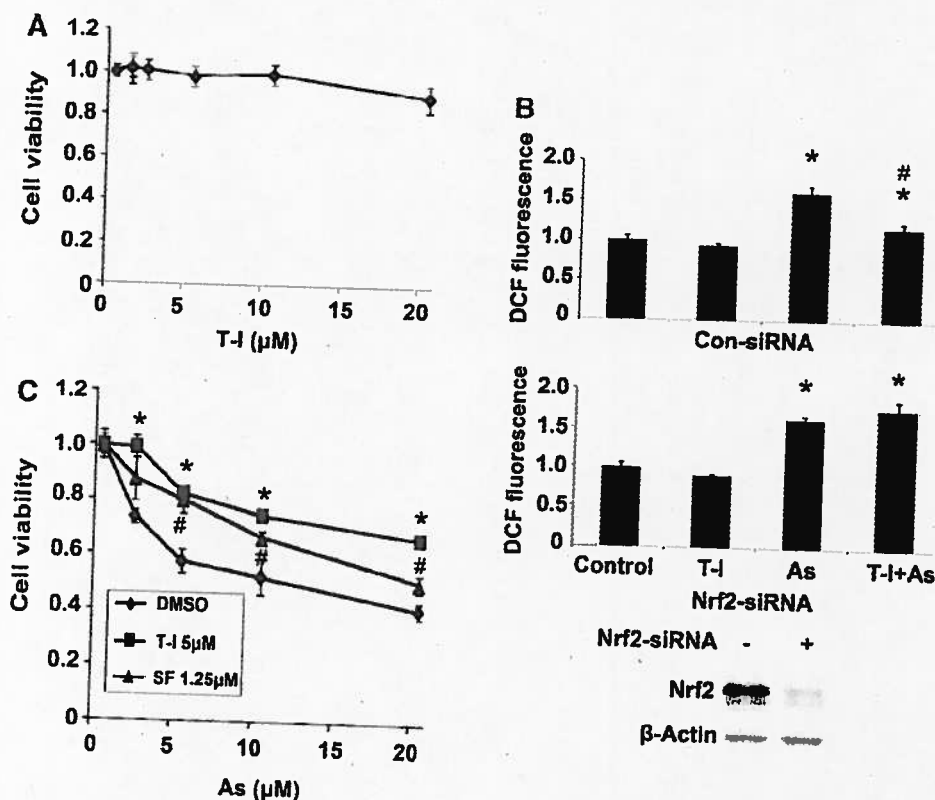
was conducted using two doses (Fig. 3B). A significant increase in Nrf2 protein level was observed as early as 2 h and persisted up to 12 h at both doses (Fig. 3B). Again, there was no effect on Keap1 protein levels. Nrf2 protein levels returned to basal levels by 24 h (Fig. 3B), but protein levels of two Nrf2 downstream target genes, NQO1 and  $\gamma$ -GCS, displayed sustained upregulation up to 48 h (Fig. 3B). Consistent with the notion that T-I activates the Nrf2 pathway by stabilizing Nrf2 proteins, the mRNA level of Nrf2 did not change upon treatment with T-I or SF (Fig. 3C). However, mRNA levels of NQO1 and GCLM, were significantly induced by T-I treatment and the fold of their induction was comparable to that elicited by SF (Fig. 3C). Taken together these results indicate that T-I activates expression of Nrf2 target genes by upregulation of Nrf2 at the protein level in lung epithelial cells.

Next, we examined the feasibility of using T-I for cytoprotection against As(III)-mediated toxicity. First, the cytotoxicity of T-I was tested in HBE cells treated with several doses of T-I for 28 h. Ninety percent of the cells remained viable after 20  $\mu$ M T-I treatment (Fig. 4A). Therefore, a non-toxic dose (5  $\mu$ M) was chosen for the subsequent protection assays. Reactive oxygen species (ROS) and cell viability were measured after HBE cells were challenged by As(III) with or without T-I pretreatment. As(III) treatment increased ROS levels, whereas T-I itself did not enhance ROS (Fig. 4B, top panel). Cotreatment with As(III) and T-I suppressed As(III)-induced ROS levels (Fig. 4B, top panel). However, this T-I-mediated suppression of ROS was not observed in cells where Nrf2 expression was blocked by Nrf2-siRNA, indicating that the protection conferred by T-I is Nrf2-dependent (Fig. 4B, bottom panel). For the cytotoxicity protection assay, cells were exposed to several doses of As(III) in combination with either dimethyl sulfoxide (DMSO), 5  $\mu$ M T-I, or 1.25  $\mu$ M SF (a

dose effective in inducing Nrf2 in HBE cells, data not shown) and cell viability was measured after 48 h. Remarkably, T-I and SF treatment improved cell viability in response to As(III) treatment (Fig. 4C). Taken together, these data indicate that T-I is able to maintain the cellular redox balance upon As(III) challenge and protects cells against As(III)-induced cell death.

#### *T-I activates the Nrf2 signaling pathway and attenuates As(III)-induced inflammation in lungs from Nrf2<sup>+/+</sup> but not Nrf2<sup>-/-</sup> mice*

To further explore the potential cytoprotective activity of T-I in a relevant animal model, we tested T-I in an *in vivo* As(III) inhalation model recently established by our team. First, a pilot study was carried out to test the best treatment regimen (dose and injection frequency) that results in repeated activation of Nrf2-dependent response. We demonstrated that systemic delivery of T-I (10 mg/kg, i.p., every 48 h) is effective in upregulating pulmonary protein levels of Nrf2 and Nrf2 target genes ( $\gamma$ -GCS and NQO1), in Nrf2<sup>+/+</sup> but not from Nrf2<sup>-/-</sup> mice, as measured by both immunohistochemistry (IHC) (Fig. 5A) and immunoblot analysis (Fig. 5B). Next, feasibility of T-I-based tissue protection was examined. Mice were given either corn oil (control) or T-I (10 mg/kg, dissolved in corn oil) through i.p. injection every other day, while breathing in As(III)-containing dust for 15 consecutive days. Hematoxylin and eosin (HE) staining revealed infiltration of inflammatory cells and alveolar septal thickening in the lungs of both Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice exposed to dust containing As(III) (Fig. 6A, HE panel). T-I injection did not affect the lungs of mice in either genotype (Fig. 6A, HE panel). Importantly, in mice exposed to As(III)

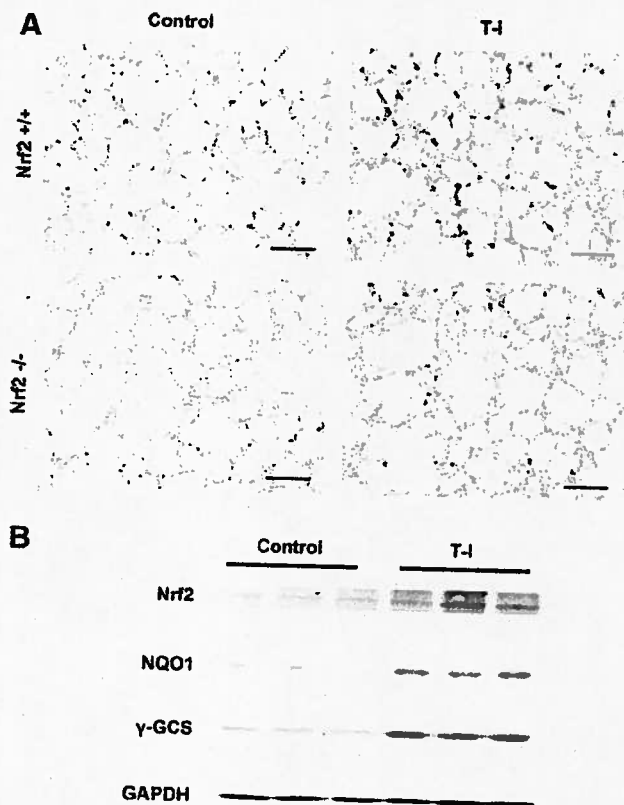


**FIG. 4. T-I protects HBE cells against As(III) cytotoxicity.** (A) Cell viability was measured in HBE cells treated with several doses of T-I at 28 h. (B) HBE cells were transfected with either control-siRNA or Nrf2-siRNA for 24 h. HBE cells were then pretreated with DMSO or T-I (5  $\mu$ M) for 4 h before the treatment with As(III) (10  $\mu$ M) for an additional 24 h. DCF-based fluorescence was measured using flow cytometry. Data were expressed as means  $\pm$  SD (\* $p$  < 0.05 DMSO group vs. As(III)-treated group; # $p$  < 0.05 As(III)-treated group vs. As(III) + T-I-treated group). (C) Cells were left untreated or pretreated with 5  $\mu$ M T-I for 24 h before addition of the indicated dose of As(III). Cell viability was measured 48 h after addition of As(III). Data are expressed as means  $\pm$  SD (\* $p$  < 0.05 DMSO group vs. T-I-treated group; # $p$  < 0.05 DMSO group vs. SF-treated group). DMSO, dimethyl sulfoxide.

and cotreated with T-I, pulmonary pathological alterations were reduced in Nrf2<sup>+/+</sup> mice, whereas no improvement was observed in Nrf2<sup>-/-</sup> lungs (Fig. 6A, HE panel). Furthermore, IHC analysis for 8-hydroxy-2'-deoxyguanosine (8-OHdG) was performed to detect As(III)-induced oxidative DNA damage. As(III) enhanced 8-OHdG staining in both Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice (Fig. 6A, 8-OHdG panel). In contrast, T-I itself did not have any effect; rather cotreatment with T-I and As(III) suppressed 8-OHdG staining in Nrf2<sup>+/+</sup>, but not in Nrf2<sup>-/-</sup> mice (Fig. 6A, 8-OHdG panel). These results indicate that T-I protects against arsenic-mediated pulmonary damage through activation of the Nrf2 pathway. To further support the data obtained, analysis of bronchoalveolar lavage (BAL) fluid for total and distinct inflammatory cell types was conducted. As(III) increased inflammatory cell infiltration as assessed by the number of total cells as well as the number of macrophages, neutrophils, or lymphocytes in both Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice (Fig. 6B). T-I administration in unchallenged mice did not change any of the inflammatory cell numbers in either genotype. However, T-I treatment decreased As(III)-induced inflammatory cell infiltration (total, macrophages, lymphocytes) only in Nrf2<sup>+/+</sup> mice, but not in Nrf2<sup>-/-</sup> mice (Fig. 6B). A similar effect was observed with neutrophils but did not reach to the level of statistical significance (Fig. 6B). These data suggest that T-I can decrease

pulmonary inflammatory response associated with As(III) exposure through activation of the Nrf2-dependent defensive mechanism.

As expected, immunoblot analysis confirmed increased protein levels of Nrf2, NQO1,  $\gamma$ -GCS in the lungs of Nrf2<sup>+/+</sup> mice exposed to the single or combined action of T-I and As(III) at the time of tissue collection at 15 day post-treatment (Fig. 7A). On the other hand, protein levels of Nrf2, NQO1, and  $\gamma$ -GCS were not affected by any of the treatments in Nrf2<sup>-/-</sup> mice (data not shown). Since NF- $\kappa$ B is a common signaling pathway regulating inflammatory response, protein levels of phosphorylated-p65 (p-p65) versus p65 were assessed as a measurement of NF- $\kappa$ B activation in both Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice. A significant increase in p-p65 protein level but not p65 was observed in the lung of both Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice when treated with As(III) (Fig. 7A). T-I alone did not affect p-p65 and p65 protein levels in both genotypes (Fig. 7A). When Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice were treated with both T-I and As(III), p-p65 protein levels slightly decreased when compared to the As(III) alone treated group in the lungs of Nrf2<sup>+/+</sup> mice but not Nrf2<sup>-/-</sup> mice. Independent ELISA-based analysis with interleukin (IL)-6 and TNF- $\alpha$  confirmed the As(III)-induced activation of the NF- $\kappa$ B signaling pathway and its attenuation by T-I administration that only occurred in Nrf2<sup>+/+</sup>; however, suppression of IL-6



**FIG. 5. Pulmonary Nrf2 activation by systemic administration of T-I.** *Nrf2*<sup>+/+</sup> and *Nrf2*<sup>-/-</sup> mice were i.p. injected with 10 mg/kg T-I. Mice were sacrificed at 48 h postinjection and the lung was isolated. (A) Lung tissue sections were subjected to IHC analysis with anti-Nrf2 antibodies ( $n=3$  in each group; one representative tissue section is shown per group). Scale bar: 100  $\mu$ m. (B) Lung tissue lysates from *Nrf2*<sup>+/+</sup> mice were subjected to immunoblot analysis with the indicated antibodies. Each lane contains a lung tissue sample from individual mice. Note: immunoblot analysis results were not included with tissues from *Nrf2*<sup>-/-</sup> mice because low expression levels of Nrf2, NQO1, and  $\gamma$ -GCS. IHC, immunohistochemistry;  $\gamma$ -GCS,  $\gamma$ -glutamylcysteine synthetase.

levels by T-I was not statistically significant (Fig. 7B). Participation of CD4<sup>+</sup> T cells, including Th1 and Th2 cells, in the As(III)-mediated inflammation response has recently been revealed (11, 12, 62). Therefore, we employed real-time reverse transcription-polymerase chain reaction (RT-PCR) to detect the mRNA levels of Th2 cytokines (IL-13 and IL-4) and Th1 cytokines (IL-2 and interferon gamma [IFN $\gamma$ ]) (Fig. 8). Indeed, As(III) upregulated IL-13 and IL-4 mRNA levels in both *Nrf2*<sup>+/+</sup> and *Nrf2*<sup>-/-</sup> mice, and T-I treatment alone did not affect any of the cytokines. However, when As(III) was administered together with T-I, the As(III)-mediated increase in IL-13 was suppressed only in *Nrf2*<sup>+/+</sup> lungs (Fig. 8). For IL-4, a similar effect was observed but did not reach the level of statistical significance (Fig. 8). Since IL-13 stimulates airway fibrosis largely through activation of transforming growth factor beta (TGF- $\beta$ ) (31, 34), the mRNA level of TGF- $\beta$  was also assessed. Indeed, TGF- $\beta$  mRNA levels increased when treated with As(III), which was suppressed by T-I coadministration in *Nrf2*<sup>+/+</sup> mice only, but did not reach the level of statistical

significance (Fig. 8). Conversely, As(III) decreased IL-2 and IFN $\gamma$  mRNA levels in both *Nrf2*<sup>+/+</sup> and *Nrf2*<sup>-/-</sup> lungs (Fig. 8), and T-I treatment alone did not affect IL-2 and IFN $\gamma$ . Again, when treated in conjunction with As(III), T-I restored mRNA levels of IL-2 and IFN $\gamma$  in *Nrf2*<sup>+/+</sup> mice, but not in *Nrf2*<sup>-/-</sup> mice (Fig. 8). Furthermore, monocyte chemoattractant protein-1 (MCP-1) mRNA levels increased in response to As(III) in both genotypes. However, T-I treatment suppressed the As(III)-mediated response only in *Nrf2*<sup>+/+</sup> mice (Fig. 8). This result agrees with the enhanced inflammatory cell infiltration in response to As(III) exposure and its Nrf2-dependent suppression by T-I as observed by BAL cell counting (Fig. 6B). Collectively, these results suggest that As(III)-induced pulmonary inflammatory pathology and pathogenic Th2 cytokine response can be suppressed by systemic delivery of the novel Nrf2 activator T-I.

## Discussion

Environmental and occupational exposure to As(III) represents an unresolved major public health concern affecting large populations on a global scale. An urgent need exists for the development of strategies that prevent or limit tissue damage and pathologies associated with acute and chronic As(III) exposure. Tanshinones, phenanthrenequinone constituents of the Chinese medicinal herb Danshen (*S. miltiorrhiza*), have recently emerged as potent antioxidant, anti-inflammatory, and cytoprotective factors showing efficacy in cancer (14, 35), cardiovascular disease (29, 30), ischemia reperfusion injury (1), and hepatic fibrosis (52).

By comparing the ability of four major tanshinones in inducing Nrf2 activity, we identified that both T-I and DHT are Nrf2 activators. Since T-I elicits less cytotoxicity than DHT, T-I was further characterized for its mechanistic action of Nrf2 activation and for its potential therapeutic application. Our experiments demonstrate for the first time that T-I induces the Nrf2-dependent response primarily by hindering its ubiquitination and degradation of Nrf2, and thus, stabilizing Nrf2 protein levels in a Keap1-C151-dependent manner (Figs. 1 and 2). According to this result, T-I is a canonical Nrf2 activator. More importantly, the potential therapeutic use of T-I as a novel Nrf2 inducer has not been reported until now. Unlike many experimental Nrf2-inducers with undefined or unfavorable pharmacokinetic profiles, tanshinones are investigational drugs currently in advanced stages of clinical development in human patients with cardiovascular diseases and other indications (clinicaltrials.gov: NCT01452477; NCT01637675). Therefore, in this study we investigated the therapeutic potential of T-I in protecting against arsenic toxicity using both HBE cells *in vitro* and a whole animal inhalation model relevant to human low doses of environmental exposure to arsenic *in vivo*.

In HBE cells exposed to As(III), T-I upregulated the Nrf2-orchestrated gene expression and displayed pronounced cytoprotective activity, an effect associated with pharmacological activation of Nrf2 by T-I (Figs. 3 and 4). T-I conferred cellular protection by maintaining cellular redox homeostasis under As(III) challenge and thus, enhancing cell viability (Fig. 4). Coordinated induction of the Nrf2-mediated cellular defense response, including upregulation of cellular antioxidant, phase II detoxifying enzymes, transporters and many other cytoprotective proteins, may contribute to the T-I-mediated protection observed in response to As(III) exposure.

In an *in vivo* As(III) dust inhalation model conducted in Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice, preventive efficacy of systemic administration of T-I was only observed in Nrf2<sup>+/+</sup> mice, consistent with the notion that T-I-mediated tissue protection is derived from activation of the Nrf2 pathway (Fig. 6). The results obtained using HE staining, 8-OHdG IHC and BAL cell counting indicate that inhaled As(III) dust caused similar severity of pulmonary oxidative damage and inflammation (except lymphocyte infiltration shown in Fig. 6B, lymphocyte panel) in both Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice as judged by infiltration of inflammatory cells and alveolar septal thickening (Fig. 6). These results are coherent with our previous study demonstrating that in mice given drinking water contaminated with As(III), lung inflammation occurred irrespective of Nrf2 genotype (25). The reason that Nrf2<sup>+/+</sup> mice did not show reduced inflammation compared to Nrf2<sup>-/-</sup> mice may be due to the extremely low basal level of Nrf2 in the lung. However, administration of T-I attenuated inflammatory events in the lung of Nrf2<sup>+/+</sup> mice without showing therapeutic effects in Nrf2<sup>-/-</sup> mice. Similarly, protein levels of p-p65, an inflammatory marker indicative of NF- $\kappa$ B activation, also increased upon As(III) exposure irrespective of Nrf2 status, but was suppressed by T-I administration only in Nrf2<sup>+/+</sup> mice (Fig. 7). These results strongly suggest that activation of Nrf2 by T-I plays a critical role in protecting lungs from As(III)-induced inflammation and toxicity.

Th2 polarized immune response is implicated in the pathology of allergic diseases (39). When assessing the potential participation of CD4<sup>+</sup> T cells, including Th1 and Th2 cells, in As(III)-induced lung inflammation using real-time RT-PCR-based profiling of cytokine expression, we observed an As(III)-induced Th2 polarized cytokine response as evidenced from the upregulation of IL-13 and IL-4 mRNA in lung tissue (Fig. 8). In addition to IL-13 and IL-4, proinflammatory cytokines, including TGF- $\beta$  and MCP-1 were also increased by As(III) inhalation. In contrast, other proinflammatory cytokines, such as Th1 cytokines (IL-2 and IFN $\gamma$ ) displayed an As(III)-induced decrease, changes that were observed in response to As(III) exposure irrespective of Nrf2 status. This observation suggests that the inflammatory response to As(III) is not Nrf2-dependent. However, when mice were treated with T-I in combination with As(III) exposure, the mRNA levels of all cytokines were restored in Nrf2<sup>+/+</sup> mice only (Fig. 8), suggesting that Nrf2 plays a protective role against the As(III)-induced inflammatory response by acting as an immune modulator. However, the detailed mechanisms by which Nrf2 modulates the immune response require further investigation.

There is mounting evidence suggesting that As(III) induces ROS by depleting GSH or by damaging mitochondria (33, 47). Therefore, it was proposed that As(III) may indirectly activate the Nrf2 pathway by increasing ROS (36). However, the doses

used in these earlier experiments showing generation of ROS in response to As(III) are extremely high. On the other hand, low environmentally relevant doses of arsenic do not seem to cause detectable changes in the intracellular redox status (33). Therefore, it is unlikely that the Nrf2-mediated protection against arsenic toxicity solely relies on the Nrf2-dependent antioxidant response that neutralizes ROS. Recently, we found that at low concentrations, arsenic is able to block autophagic flux, resulting in accumulation of autophagosomes where Keap1 and p62 proteins aggregate. Nrf2 activation by canonical Nrf2 activators, such as SF and tBHQ completely reversed autophagosomal accumulation in response to As(III), suggesting that canonical Nrf2 activators can be used to block As(III)-mediated autophagy deregulation (manuscript accepted to *Molecular and Cellular Biology*). The possible mechanisms of Nrf2 activator-mediated protection against arsenic toxicity are still not clear, although many compounds, such as SF, CA, tBHQ, and lipoic acid have been shown to protect against arsenic toxicity both *in vitro* and *in vivo* (33). Shinkai *et al.* demonstrated that activation of Nrf2 by SF reduced intracellular accumulation of As(III), and thus, reduced As(III) toxicity (48). The authors have suggested that upregulation of certain Nrf2 target genes, such as  $\gamma$ -GCS, GST isoforms and MRP1 enhance the excretion of arsenic (48). Therefore, it is highly possible that T-I alleviates the arsenic effect by reducing the intracellular concentration of arsenic through reduced uptake and enhanced export, which requires further investigation.

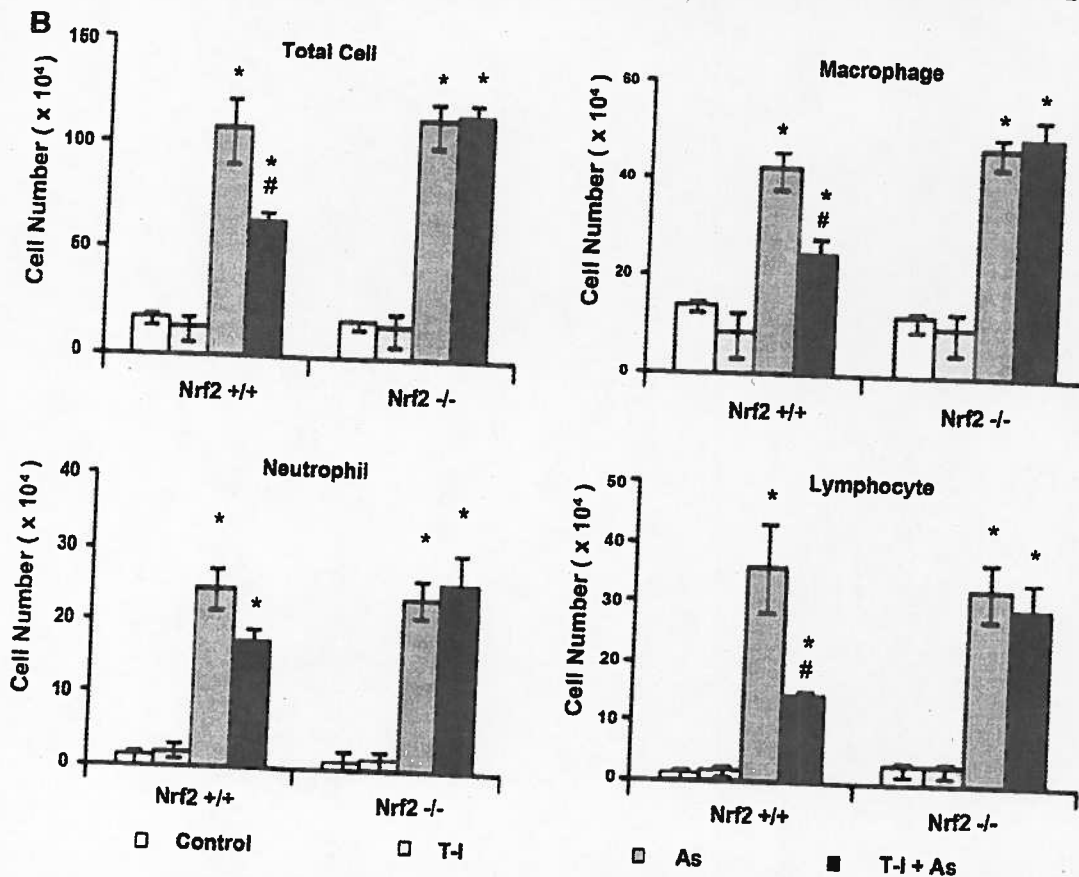
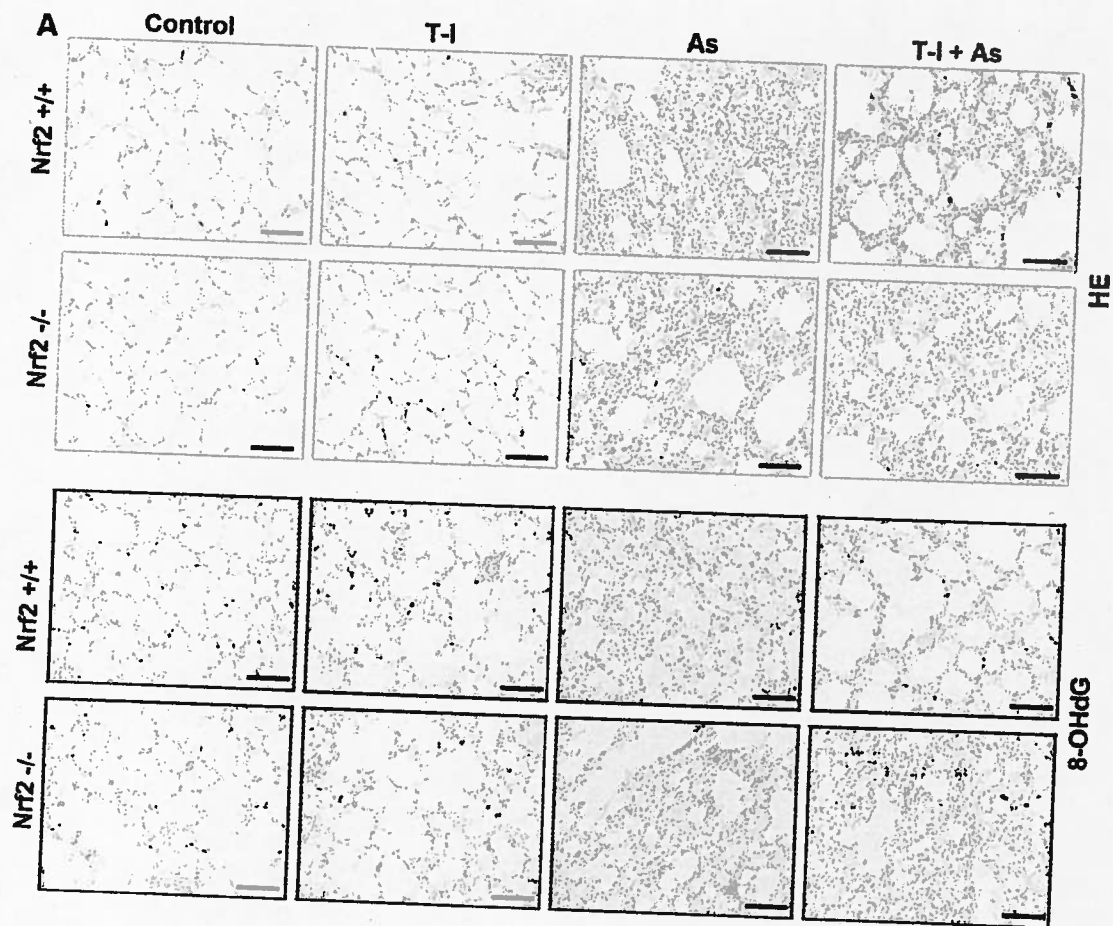
Taken together, our experiments demonstrate the feasibility of preventing As(III)-induced lung inflammation by systemic administration of T-I, a novel canonical Nrf2 activator characterized for the first time in this study. The T-I-mediated intervention may also prove to be efficacious for other types of environmental insults and may also confer protection against tissue damage in other organs.

## Materials and Methods

### Chemicals and cell culture

T-I and As(III) (NaAsO<sub>2</sub>) were purchased from Sigma, and SF was purchased from Santa Cruz. Human bronchial epithelium cells 16HBE14o (HBE) were obtained from California Pacific Medical Center, San Francisco. HBE cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS; Atlanta Biological), 5% L-glutamine, and 0.1% gentamycin (Invitrogen). Human MDA-MB-231 breast carcinoma cells, purchased from ATCC, were cultured in MEM supplemented with 10% FBS, 5% L-glutamine, 0.1% gentamycin, 2 mM HEPES and 6 ng/ml bovine insulin (Invitrogen). All mammalian cells were incubated at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

**FIG. 6. T-I attenuates As(III)-induced pathological alterations and inflammatory cell infiltration in lungs from Nrf2<sup>+/+</sup> but not Nrf2<sup>-/-</sup> mice.** Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice received systemic delivery of corn oil or T-I (10 mg/kg, i.p., every 48 h) for 15 days. During these 15 days, mice were also exposed to As(III)-containing dusts for 30 min everyday. (A) HE staining and IHC of 8-OHdG of lavaged lung tissue sections from Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice. A representative image of the lung tissue from each group is shown. (B) Cell differential analysis was performed on the BAL cells from each mouse. After staining, at least 200 cells were counted under a microscope. The absolute number of total cells, macrophages, neutrophils, or lymphocytes was plotted. Results are expressed as means  $\pm$  SD ( $n=5$ ) (\* $p<0.05$  DMSO group vs. As(III)-administrated group;  $p<0.05$  As(III)-administrated group vs. As(III)+T-I-administrated group). BAL, bronchoalveolar lavage; HE, hematoxylin and eosin; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.



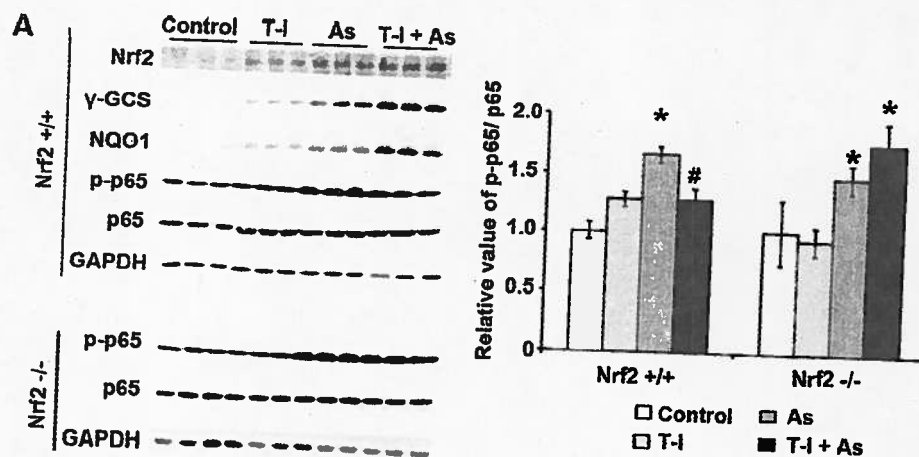


FIG. 7. T-I activates the Nrf2 signaling pathway attenuating As(III)-induced inflammation in lungs from *Nrf2*<sup>+/+</sup> but not *Nrf2*<sup>-/-</sup> mice. (A) Lung tissue lysates (three mice per group) were subjected to immunoblot analysis (left panel). The intensity of bands was quantified and the relative value of p65/p65 was plotted (right panel). (B) The amount of IL-6 and TGF-β in the BAL fluid was measured by ELISA. Results are expressed as means ± SD (*n* = 5) (\**p* < 0.05 control group vs. As(III)-administrated group; #*p* < 0.05 As(III) group vs. As(III) + T-I-administrated group). IL, interleukin; TGF-β, transforming growth factor beta.

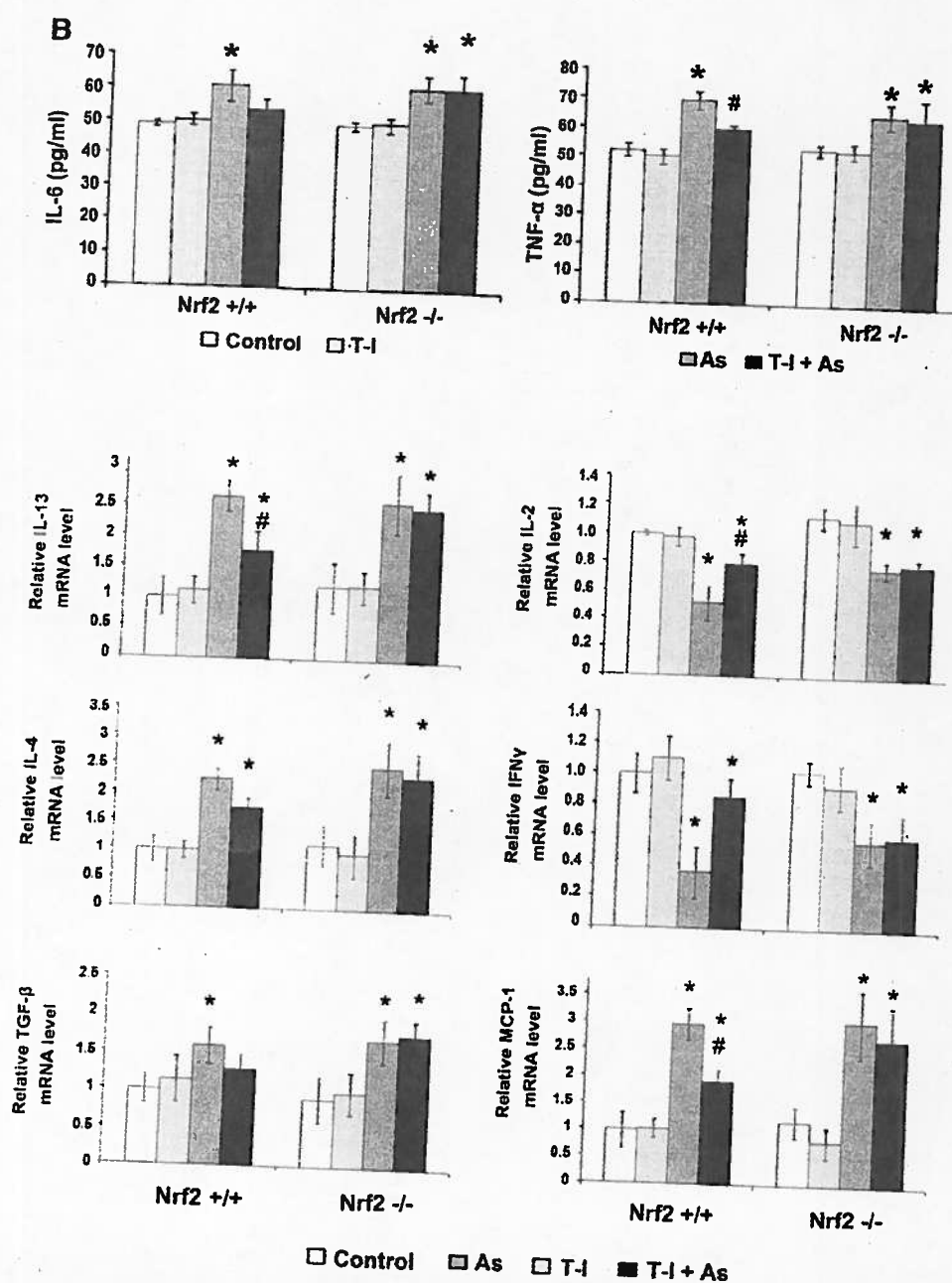


FIG. 8. T-I restores As(III)-induced immuno-proinflammatory cytokine production in lungs from *Nrf2*<sup>+/+</sup> but not *Nrf2*<sup>-/-</sup> mice. Relative mRNA expression of IL-13, IL-4, TGF-β, IL-2, IFNγ, and MCP-1 was measured by real-time RT-PCR. mRNAs extracted from three mice per group were used to run RT-PCR in duplicate and the mean ± SD was calculated (*n* = 3) (\**p* < 0.05 control group vs. As(III)-administrated group; #*p* < 0.05 As(III) group vs. As(III) + T-I-administrated group). MCP-1, monocyte chemoattractant protein-1; IFNγ, interferon gamma.

*Transfection of siRNA, cDNA, and luciferase reporter gene assay*

Transfection of cDNA was performed using Lipofectamine Plus (Invitrogen) and HiPerfect was used for transfection of siRNA, both were used according to the manufacturer's instructions. Nrf2-siRNA (SI03187289) and Keap1-siRNA (SI03246439) were purchased from Qiagen. Activation of Nrf2-dependent transcriptional activity by test compounds was examined as previously published (54). MDA-MB-231 cells were transfected with the mGST-ARE firefly luciferase reporter plasmid together with expression plasmids for Nrf2, Keap1, and renilla luciferase, an internal control, using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. At 24 h post-transfection, cells were treated with the test compounds for 16 h before cell lysis for analysis of reporter gene activity. Reporter assays were performed using Promega dual-luciferase reporter gene assay system. All samples were run in triplicate for each experiment and the data represent the means of three independent experiments.

*Cell viability*

As(III)-induced toxicity was measured by functional impairment of the mitochondria using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma). Approximately  $5 \times 10^4$  HBE cells per well were seeded in a 96-well plate and pretreated with DMSO, 5  $\mu$ M T-I or 1.25  $\mu$ M SF for 24 h. Cells were then cotreated with the indicated concentrations of As(III) (up to 20  $\mu$ M) for 48 h. Twenty microliters of 2 mg/ml MTT was directly added to the cells. After incubation (37°C, 0.5–3 h), the plate was centrifuged and the medium was removed by aspiration. One hundred microliters of isopropanol/HCl was added to each well and was shaken at room temperature to dissolve the crystals. Absorbance was measured at 570 nm using the Synergy 2 Multi-Mode Microplate Reader (Biotek). All samples were run in triplicate for each experiment and the data represent the means of three independent experiments.

*ROS detection*

Cells were pretreated with DMSO (control) or 5  $\mu$ M T-I for 4 h before the treatment with 10  $\mu$ M As(III) for an additional 24 h. Cells were then washed with phosphate-buffered saline (PBS) and fresh medium containing 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) (Sigma; 10  $\mu$ g/ml final concentration) was added. Plates were incubated for 20–60 min at 37°C. Cells were washed twice with PBS, trypsinized, washed again with PBS, and resuspended in PBS to  $\sim 10^6$  cells per ml. Fluorescence was measured using flow cytometry with excitation at 488 nm and emission at 515–545 nm. All steps were handled in the dark.

*Antibodies, immunoblot analysis, ubiquitination assay, and protein half-life*

Antibodies for Nrf2, Keap1, NQO1,  $\gamma$ -GCS and  $\beta$ -actin were purchased from Santa Cruz. Cells were harvested in sample buffer (50 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 100 mM dithiothreitol (DTT), and 0.1% bromophenol blue). After sonication, cell lysates were electrophoresed through an SDS-polyacrylamide gel and

subjected to immunoblot analysis. For ubiquitination assay, MDA-MB-231 cells were cotransfected with expression vectors for HA-tagged ubiquitin, Nrf2 and Keap1, cells were treated with either 5  $\mu$ M SF or T-I along with 10  $\mu$ M MG132 for 4 h. Cells were harvested in buffer containing 2% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM DTT and immediately boiled. The lysates were then diluted fivefold in buffer lacking SDS and incubated with an anti-Nrf2 antibody. Immunoprecipitated proteins were analyzed by immunoblot with an antibody against the HA epitope (Santa Cruz). To measure the half-life of Nrf2, MDA-MB-231 cells were either left untreated or treated with 5  $\mu$ M T-I for 4 h. 50  $\mu$ M cycloheximide was added to block protein synthesis. Total cell lysates were collected at different time points and subjected to immunoblot analysis with an anti-Nrf2 antibody. The relative intensity of the bands was quantified using the ChemiDoc CRS gel documentation system and Quantity One software from BioRad.

*mRNA extraction and real-time RT-PCR*

RT-PCR was done on total mRNA extracted from cells using TRIzol (Invitrogen). Equal amounts of mRNA were used to generate cDNA using the Transcriptor First Strand cDNA synthesis kit purchased from Roche. RT-PCR procedures and primer sequences of Nrf2, NQO1, GCLM, and GAPDH were described previously and the LightCycler 480 system was used (Roche) (50).

Quantification of cDNA amount for mIL-13, mIL-4, mIL-2, mIFN $\gamma$ , mTGF- $\beta$ , mMCP-1, and m $\beta$ -actin in each tissue sample was performed with KAPA SYBR FAST qPCR Kit (Kapa Biosystems). All primer sets were designed with Primer 3 online free software. And the primers were synthesized by Sigma.

mIL-13: forward (caagaccagactccctctgtg) and reverse (aggccatgaatatcctctgtg);  
mIL-4: forward (ccaagtgcttcgcatattt) and reverse (atc-gaaaagcccgaagagat);  
mIL-2: forward (aagctctacagcggaagcac) and reverse (atcctggggagtttcagggtt);  
mIFN $\gamma$ : forward (actggcaaaaggatggtgac) and reverse (gctgatggcctgattgtctt);  
mTGF- $\beta$ : forward (gactctccacctgaagacc) and reverse (gactggcgagccttagtttg);  
mMCP-1: forward (cccaatgagtaggctggaga) and reverse (tctggaccattccttcttg);  
m $\beta$ -actin: forward (aaggccaacctgaaaagat) and reverse (gtggtacgaccagaggcatac).

The real-time PCR conditions used were the following: one cycle of initial denaturation (95°C for 3 min), 40 cycles of amplification (95°C for 10 s, 60°C for 20 s and 72°C for 5 s), melting curve (95°C for 5 s, 65°C for 1 min and 97°C continuous), and a cooling period (40°C for 30 s). Mean crossing point (Cp) values and standard deviations (SD) were determined. Cp values were normalized to the respective crossing point values of the m $\beta$ -actin reference gene. Data are presented as a fold change in gene expression compared to the control group. All reporter gene and RT-PCR analysis were repeated in three independent experiments and in duplicates. Data are all shown as means  $\pm$  SD.

### Animals and treatments

Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice were obtained by breeding Nrf2 heterozygous mice. All animals received water and food *ad libitum*. Eight-week-old mice were used for the experiment. Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice were randomly allocated into four groups ( $n=5$  per group): (i) control (corn oil); (ii) T-I (10 mg/kg, dissolved in corn oil); (iii) As(III); (iv) As(III) + T-I; T-I was administered through intraperitoneal (i.p.) injection every other day for 15 days. During these 15 days mice were also exposed to 4.8 mg/m<sup>3</sup> of the synthetic dust containing 10% As(III) for 30 min/day (62). All 40 mice survived As(III)-dust exposure and/or T-I injections. The dose of T-I was tested initially in a pilot study to ensure Nrf2 was activated up to 48 h after i.p. injection.

### BAL and lung tissue collection

After treatment, mice were euthanized and lungs were isolated by carefully opening the thoracic cavity. BAL fluid was obtained by lavaging the lung with 0.5 ml PBS three times. The BAL fluid was centrifuged at 1500 rpm for 8 min at 4°C. Cell pellets were pooled, washed, and resuspended in PBS. Total cell counts were determined using standard hematologic procedures (63). Cytospins of BAL cells were prepared and slides were stained with a HEMA3 STAT PACK kit (Fisher Scientific Company). Macrophages, neutrophils, and lymphocytes were identified using the standard morphologic criteria. A minimal of 200 cells was examined. The means  $\pm$  SD were obtained by analyzing three batches of BAL fluid, each from individual mice in the same group. The supernatant of the first injection was stored at -80°C until used for an ELISA. Lungs were then collected and divided into two parts: one part was frozen in liquid nitrogen for total RNA extraction and protein analysis. The other part was fixed in 10% buffered formalin to be embedded in paraffin and cut into 4  $\mu$ m sections for histological and immunochemical analyses.

### HE staining and IHC

Tissue sections were stained with HE for pathological examination. IHC analysis was performed as previously described (25). A monoclonal antibody for 8-OHdG was purchased from Trevigen. Briefly, antigen retrieval of formalin-fixed paraffin-embedded tissue sections was carried out by microwave heating for 7 min at the highest setting to allow the retrieval solution to boil. Next, the sections were microwaved for 10 min at the lowest setting to maintain the retrieval solution at the boiling temperature. The retrieval solution contains 1  $\times$  TBS with 0.1% Tween 20 (TBS-T) in 1 mol/L sodium citrate. After antigen retrieval, tissue sections were exposed to 3.5 M HCl for 15 min at room temperature and washed in TBS-T. Subsequently, tissue sections were treated with 0.3% peroxidase to quench endogenous peroxidase activity. Tissue sections were incubated with 5% normal goat serum for 30 min followed by 2 h incubation with an Nrf2 antibody at 1:100 dilution at room temperature. Sections were then incubated with a biotinylated goat anti-rabbit secondary antibody for 1 h. The ABC kit (Vector Laboratories) was then used according to the manufacturer's instructions. Finally, tissue sections were developed for 30 s using the 3,3'-diaminobenzidine staining kit (Dako), and counterstained with hematoxylin.

### ELISA of cytokines in BAL fluid

The ELISA was purchased from eBiosciences and used according to the manufacturer's instructions. Briefly, the plate was coated with 100  $\mu$ l capture antibody in coating buffer per well and incubated overnight at room temperature. The plate was washed with 250  $\mu$ l wash buffer, blocked with 200  $\mu$ l of the assay diluents, and incubated at room temperature for 1 h. A 100  $\mu$ l of the BAL fluid was added and incubated at room temperature for 2 h. One hundred microliters detection antibody was then added to each well and incubated for 1 h at room temperature. Subsequently, 100  $\mu$ l avidin-HRP was added and the plate was incubated for 30 min at room temperature. One hundred microliters of the substrate solution was added to each well and incubated for 15 min at room temperature and then 50  $\mu$ l of the stop solution was added to stop the reaction. The plate was then read at 450 nm and analyzed. The ELISA was performed in triplicate. Serial dilutions of standards were also used to obtain a standard curve.

### Statistics

Results are presented as the mean  $\pm$  SD of at least three independent experiments performed in duplicate or triplicate each. Statistical tests were performed using SPSS 10.0. Unpaired Student's *t*-tests were used to compare the means of two groups. One-way analysis of variance was applied to compare the means of three or more groups.  $p < 0.05$  was considered to be significant.

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### Author Disclosure Statement

The authors declare no conflicts of interest.

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**Abbreviations Used**

8-OHdG = 8-hydroxy-2'-deoxyguanosine  
 ARE = antioxidant response element  
 As(III) = arsenic  
 BAL = bronchoalveolar lavage  
 CT = cryptotanshinone  
 Cul3 = Cullin 3  
 DHT = dihydrotanshinone  
 DMSO = dimethyl sulfoxide.  
 DTT = dithiothreitol  
 FBS = fetal bovine serum  
 GAPDH = glyceride-3-phosphate dehydrogenase  
 GSH = glutathione  
 GSTs = glutathione S-transferases  
 H<sub>2</sub>DCFDA = 2',7'-dichlorodihydrofluorescein diacetate  
 HBE = human bronchial epithelial  
 HE = hematoxylin and eosin

IFN $\gamma$  = interferon gamma  
 IHC = immunohistochemistry  
 IL = interleukin  
 Keap1 = Kelch-like ECH-associated protein-1  
 MCP-1 = monocyte chemoattractant protein-1  
 MEM = minimal Eagle's medium  
 MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
 NQO1 = NAD(P)H quinone oxidoreductase  
 Nrf2 = NF-E2 p45-related factor 2  
 PBS = phosphate-buffered saline  
 ROS = reactive oxygen species  
 RT-PCR = reverse transcription-polymerase chain reaction  
 SD = standard deviation  
 SDS = sodium dodecyl sulfate  
 SF = sulforaphane  
 tBHQ = tert-butylhydroquinone  
 TGF- $\beta$  = transforming growth factor beta  
 T-I = tanshinone I  
 T-IIA = tanshinone IIA  
 $\gamma$ -GCS =  $\gamma$ -glutamylcysteine synthetase

**Bohn, Brent**

1595

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**From:** Mendez Jr, William <William.MendezJr@icfi.com>  
**Sent:** Tuesday, January 14, 2014 1:53 PM  
**To:** Powers, Christina  
**Subject:** MOA call tomorrow at 9:00

Just wanted to make sure that my acceptance went through. For some reason, the meeting did not make it onto my calendar.

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